THE DEVELOPMENT OF AN EFFECTIVE VACCINE AGAINST *CHLAMYDIA*: UTILISATION OF A NON-TOXIC MUCOSAL ADJUVANT TO GENERATE A PROTECTIVE MUCOSAL RESPONSE

Connor Patrick O'Meara

Bachelor of Science – Biological Science (Hons)

Institute of Health and Biomedical Innovation (IHBI), School of Biomedical Sciences, Faculty of Health

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LIST OF KEYWORDS

Adjuvant, animal model, *Chlamydia*, cholera toxin, common mucosal immune system, CpG bacterial DNA, CTA1-DD, immunity, immunisation, infection, intranasal, sublingual, transcutaneous, vaccine.

ABSTRACT

Chlamydia is responsible for a wide range of diseases with enormous global economic and health burden. As the majority of chlamydial infections are asymptomatic, a vaccine has greatest potential to reduce infection and disease prevalence. Protective immunity against *Chlamydia* requires the induction of a mucosal immune response, ideally, at the multiple sites in the body where an infection can be established. Mucosal immunity is most effectively stimulated by targeting vaccination to the epithelium, which is best accomplished by direct vaccine application to mucosal surfaces rather than by injection. The efficacy of needle-free vaccines however is reliant on a powerful adjuvant to overcome mucosal tolerance. As very few adjuvants have proven able to elicit mucosal immunity without harmful side effects, there is a need to develop non-toxic adjuvants or safer ways to administered pre-existing toxic adjuvants.

In the present study we investigated the novel non-toxic mucosal adjuvant CTA1-DD. The immunogenicity of CTA1-DD was compared to our "gold-standard" mucosal adjuvant combination of cholera toxin (CT) and cytosine-phosphate-guanosine oligodeoxynucleotide (CpG-ODN). We also utilised different needle-free immunisation routes, intranasal (IN), sublingual (SL) and transcutaneous (TC), to stimulate the induction of immunity at multiple mucosal surfaces in the body where *Chlamydia* are known to infect. Moreover, administering each adjuvant by different routes may also limit the toxicity of the CT/CpG adjuvant, currently restricted from use in humans. Mice were immunised with either adjuvant together with the chlamydial major outer membrane protein (MOMP) to evaluate vaccine safety and quantify the induction of antigen-specific mucosal immune responses. The level of protection against infection and disease was also assessed in vaccinated animals following a live genital or respiratory tract infectious challenge.

The non-toxic CTA1-DD was found to be safe and immunogenic when delivered via the IN route in mice, inducing a comparable mucosal response and level of protective immunity against chlamydial challenge to its toxic CT/CpG counterpart administered by the same route. The utilisation of different routes of immunisation strongly influenced the distribution of antigen-specific responses to distant mucosal surfaces

and also abrogated the toxicity of CT/CpG. The CT/CpG-adjuvanted vaccine was safe when administered by the SL and TC routes and conferred partial immunity against infection and pathology in both challenge models. This protection was attributed to the induction of antigen-specific pro-inflammatory cellular responses in the lymph nodes regional to the site of infection and rather than in the spleen. Development of non-toxic adjuvants and effective ways to reduce the side effects of toxic adjuvants has profound implications for vaccine development, particularly against mucosal pathogens like *Chlamydia*. Interestingly, we also identified two contrasting vaccines in both infection models capable of preventing infection or pathology exclusively. This indicated that the development of pathology following an infection of vaccinated animals was independent of bacterial load and was instead the result of immunopathology, potentially driven by the adaptive immune response generated following immunisation. While both vaccines expressed high levels of interleukin (IL)-17 cytokines, the pathology protected group displayed significantly reduced expression of corresponding IL-17 receptors and hence an inhibition of signalling. This indicated that the balance of IL-17-mediated responses defines the degree of protection against infection and tissue damage generated following vaccination. This study has enabled us to better understand the immune basis of pathology and protection, necessary to design more effective vaccines.

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THESIS-ASSOCIATED PRESENTATIONS AND AWARDS

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International Congress for Mucosal Immunology (ICMI) conference, Paris, France, 2011

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ABS	Australia bureau of statistics
*act*B	β actin
ADCC	Antibody-dependent cellular cytotoxicity
ADP	Adenosine diphosphate
AID	Activation-induced cytidine deaminase
APC	Antigen-presenting cell
ARC	Animal Resource Centre
ASC	Apoptosis-associated speck-like protein containing caspase recruitment domain
ASM	Australia Society for Microbiology
ASMR	Australia Society for Medical Research
ASI	Australia Society for Immunology
ATCC	American Type Culture Collection
BAL	Bronchoalveolar lavage
BBS	Borate buffered saline
BCR	B cell receptor
BMDC	Bone marrow-derived dendritic cell
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CDC	Centres for Disease Control
cDNA	Complementary DNA

CFA	Complete Freund's adjuvant
CLR	C-type lectin receptors
CMIS	Common mucosal immune systemic
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
CPAF	Chlamydial protease-like activity factor
CpG-ODN	Cytosine-phosphate-guanosine oligodeoxynucleotide
cpm	Counts per minute
CSR	Class switching recombination
CT	Cholera toxin
Cta1	*Chlamydia*-specific T cell antigen 1
CTL	Cytotoxic lymphocyte
CVD	Cardiovascular disease
Da	Dalton
DAMP	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
DPC	Dodecylpyridinium chloride
DTH	Delayed-type hypersensitivity
EAE	Experimental allergic encephalitis
EB	Elimentary body

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope glycoprotein
ETEC	Enterotoxigenic *Escherichia coli*
FDC	Follicular dendritic cell
FOV	Fields of view
GAPDH	Glyceraldehyde-3-phosphate de hydrogenase
GC	Germinal centre
gDNA	Genomic DNA
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GTP	Guanosine-5'-triphosphate
*gus*B	Glucuronidase-β
HBSS	Hank's buffered salt solution
HEV	High endothelial venules
HIV	Human immunodeficiency virus
HPRT	Hypoanthine guanine phosphoribosyl
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HSP	Heat-shock protein
HSV	Herpes simplex virus
ICAM-1	Intercellular adhesion molecule
ICMI	International Congress for Mucosal Immunology
ICOS	Inducible co-stimulatory molecule

ICOSL	Inducible co-stimulatory ligand
IDO	Indoleamine 2,3-dioxygenase
IFA	Incomplete Freund's adjuvant
IFN	Interferon
IFNRA	Interferon receptor A
IFNγR	Interferon gamma receptor
IFU	Inclusion-forming unit
Ig	Immunoglobulin
IHBI	Institute of Health and Biomedical Innovation
IL	Interleukin
IM	Intramuscular
IMVS	Institute of Medical and Veterinary Science
IN	Intranasal
iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal
IPM	Isopropyl myristate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRF	Interferon regulatory transcription factor
IRG	Immunity-related GTPases
ISCOMs	Immune-stimulating complexes
IV	Intravenous
IVag	Intravaginal
kDa	Kilodalton

Kg	Kilogram
KO	Knockout
L	Litres
LAL	*Limulus* amebocyte lystate
LB	Luria-Bertani
LC	Langerhan's cell
LFA-1	Lymphocyte function-associated antigen
LPS	Lipopolysaccharide
LT	Heat-labile toxin
LTα	Lymphotoxin alpha
M cell	Micro-fold cell
M1	Classically-activated macrophages
M2	Alternate-activated macrophages
MAdCAM-1	Mucosal addressin cell adhesion molecule
MALT	Mucosal-associated lymphoid tissue
MBP	Maltose-binding protein
mDCs	Myeloid DCs
mg	Milligrams
MHC	Major histocompatability complex
MIF	Microimmunofluoresence
MIP	Macrophage inflammatory protein
MIVAC	Mucosal Immunobiology and Vaccine Centre
mL	Millilitres

MMP	Matrix metalloproteinase
MOMP	Major outer membrane protein
MoPN	Mouse pneumonitis
MPR	Methyl pyrolidone
MS	Multiple sclerosis
MWCO	Molecular weight cut-off
MyD88	Myeloid differentiation primary response gene 88
NAAT	Nucleic acid amplication test
NADPH	Nicotinamide adenine dinucleotide phosphate
NALT	Nasopharynx-associated lymphoid tissue
NET	Neutrophil extracellular trap
ng	Nanograms
NK	Natural killer
NLR	Nucleotide-binding oligomerisation domain-like receptor
nMOMP	Native MOMP
NO	Nitric oxide
NFκB	Nuclear factor κ B
OD	Optical density
*omp*A	Outer membrane protein A
ova	Ovalbumin
p.i	Post-infection
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells

PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Tween20
PCB	Phosphate citrate buffer
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
pg	Picograms
PID	Pelvic inflammatory disease
pIgR	Polymeric immunoglobulin receptor
PMN	Polymorphonuclear leukocytes
PRR	Pattern recognition receptor
QUT	Queensland University of Technology
RB	Reticulate body
RLR	Retinoic acid-inducible gene-like receptors
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SC	Subcutaneous
SCID	Severe-combined immunodeficiency
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SHM	Somatic hypermutaton
SL	Sublingual
SOCS	Suppressor of cytokine signalling

SPG	Sucrose-phosphate-glutamine
STAT	Signal transducer and activator of transcription
STI	Sexually transmitted infection
T3SS	Type III secretion system
TBS	Tris buffered saline
TC	Transcutaneous
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
Th0	Naive T helper
Th1	T helper type 1
Th17	T helper type 17
Th2	T helper type 2
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TMB	3,3,5,5-tetramethylbenzidine
TNF	Tumour necrosis factor
T_{reg}	Inducible T regulatory
TRIF	TIR receptor-domain-containing adapter-inducing interferon-β
T_{RM}	Tissue-resident memory T cells
trp	Tryptophan synthase
UHL	Uterine horn lavage
UV	Ultraviolet

VCAM-1	Vascular cell adhesion molecule
VL	Vaginal lavage
VLP	Virus-like particles
WHO	World Health Organization
WT	Wild type
α-GalCer	alpha-galactosylceramide
µCi	Microcurie
µg	Micrograms
µL	Microlitres

STATEMENT OF ORIGINAL AUTHORSHIP

The work contained in this thesis has not been previously submitted for a degree or diploma at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by any other person(s) except where due reference is made.

Signature: QUT Verified Signature

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CHAPTER ONE: INTRODUCTION

DESCRIPTION OF SCIENTIFIC PROBLEM

Chlamydia trachomatis is responsible for greater than 92 million new sexually transmitted infections (STIs) annually (WHO, 2012). In Australia, notification of *C. trachomatis* increased by 19% from 2009 to 2010, increasing to 74,000 new infections (ABS, 2012). Chronic chlamydial genital tract infections can lead to pelvic inflammatory disease (PID) and infertility in up to 40% of cases (WHO, 2007). The burden of caring for and treating patients with *Chlamydia*-related fertility complications costs billions of dollars each year worldwide (WHO, 2012).

Serological evidence suggests that virtually everyone will contract a *C. pneumoniae* respiratory tract infection at one point in their lifetime (Grayston, 2000). Moreover, it is reported that 75% of first infections occur in early life between the ages of 5 – 14 years (Aldous *et al.*, 1992; Miyashita, 2006). *C. pneumoniae* has been associated with the exacerbation of cardiovascular disease (CVD), asthma, chronic obstructive pulmonary disease (COPD), multiple sclerosis (MS), Alzheimer's disease and reactive arthritis (Kuo *et al.*, 1993; Clementsen *et al.*, 2002; Bachmaier and Penninger, 2005), the majority of which have multi-billion dollar healthcare expenditures and are leading causes of morbidity and mortality in most nations.

Therefore, *Chlamydia* presents a significant health burden in the community and the rapidly increasing prevalence calls for effective and immediate intervention to eradicate a growing global pandemic. Confounding the matter of infection control, between 50 – 90% of acute *Chlamydia* infections are asymptomatic (Stamm and Holmes, 1990; Grayston, 2000) and methods of screening and treating *Chlamydia* infections have largely proven ineffective at reducing infection prevalence long-term (Brunham *et al.*, 2005). Consequently, development of a vaccine is the only viable alternative.

Chlamydia is a pathogen that infects through and predominantly resides in the mucosal epithelium. To prevent the establishment of an infection requires the induction and maintenance of a mucosal immune response at the anatomical portal of entry of the invading pathogen (Igietseme and Rank, 1991). Vaccines targeted to the epithelium, without the necessity for needles, elicit mucosal immunity by stimulating

innate cell populations preconditioned to generate immune responses at mucosal surfaces (Brokstad *et al.*, 2002; Holmgren and Czerkinsky, 2005). Moreover, mucosal immunity can be induced at multiple sites in the body, like the genital and respiratory mucosa, simultaneously by utilising the correct route of immunisation.

The mucosal epithelium is constantly bombarded with foreign material like food, dust and microbes. Suppression of immune responses to innocuous exogenous antigens is a characteristic of the epithelium and crucial to avoid unnecessary inflammation and tissue damage. As tolerance is the default response to mucosal antigen exposure, any vaccine administered to the epithelium must overcome this in order to elicit robust and long-lived mucosal immunity. Unfortunately, a major obstacle to the development of an effective vaccine for *Chlamydia* and other mucosal pathogens is the lack potent adjuvants proven to elicit good responses at mucosal surfaces.

Whilst the push for research and development of new mucosal adjuvants is greater than ever, vaccine safety cannot be compromised. In 2001, Nasalflu® was withdrawn from the market due to facial paresis (Bell's palsy) in some recipients. This was later found to be due to caused by an incompatibility between the enterotoxic adjuvant (a bacterial toxin derived from *Escherichia coli*, heat-labile toxin [LT]) and the nasal route of administration (Mutsch *et al.*, 2004). Interestingly, the same adjuvant applied topically can be equally immunogenic without the inherent toxicity (McKenzie *et al.*, 2007; Frech *et al.*, 2008). This shows that utilising different routes of immunisation can limit the toxicity associated with certain adjuvants like LT, CT and CpG (Heikenwalder *et al.*, 2004; Klinman *et al.*, 2007; DeFrancesco, 2008; Lewis *et al.*, 2009), while still harnessing their adjuvanticity. Alternatively, detoxification of the enterotoxins (eg. CT), by removing the toxicity while retaining the adjuvant properties, is a growing area of research. This is because the nasal route is still of particular interest for human vaccines designed to protect against mucosal pathogens (Kiyono and Fukuyama, 2004; Brandtzaeg, 2009). The adjuvant CTA1-DD, a non-toxic derivation of CT, has been shown to be equally immunogenic as the native CT holotoxin, but devoid of the toxicity commonly associated with the

administration of enterotoxic adjuvants by the nasal route (Eriksson *et al.*, 2004; Sundling *et al.*, 2008).

HYPOTHESIS

Our hypothesis was that utilising different mucosal immunisation routes and novel non-toxic mucosal adjuvants will elicit simultaneous protection against *Chlamydia* genital and respiratory tract infections, without any harmful side effects.

SIGNIFICANCE

Eradication of infection and *Chlamydia*-related disease by vaccination has obvious economic and global health ramifications. Development of non-toxic adjuvants or effective ways to reduce the side effects of toxic adjuvants has profound implication for vaccine development, particularly against mucosal pathogens. Improving needle-free vaccine delivery would also be most beneficial in the developing world, as it is safer and more cost-effective than injectable vaccines, does not require trained personnel, facilitates rapid immunisation of large populations and enhances the population compliance critical for herd immunity (Glenn *et al.*, 2003; Giudice and Campbell, 2006).

OBJECTIVE

Immunise mice with two different vaccines (MOMP plus CTA1-DD and MOMP plus CT/CpG) delivered by three different needle-free routes (IN, SL and TC). Assess vaccine safety and the induction of mucosal responses (Chapter 4) and immunity against respiratory (Chapter 5) and genital tract (Chapter 6) challenges with *C. muridarum*.

AIMS

1. Quantify systemic and mucosal immune responses in the genital and respiratory tracts following immunisation via IN, SL and TC routes with the MOMP plus CTA1-DD or CT/CpG (Chapter 4).
2. Assess the level of protective immunity against a respiratory tract infection and associated disease following immunisation via IN, SL and TC routes with the MOMP plus CTA1-DD or CT/CpG (Chapter 5).
3. Assess the level of protective immunity against a genital tract infection and associated disease following immunisation via IN, SL and TC routes with the MOMP plus CTA1-DD or CT/CpG (Chapter 6).

CHAPTER TWO: LITERATURE REVIEW

CHLAMYDIA

Chlamydiae are Gram negative obligate intracellular pathogens with a unique biphasic lifecycle, characterised by two functionally and morphologically distinct forms, i.e. the inert infectious extracellular elementary body (EB) and the metabolically active non-infectious intracellular reticulate body (RB) (Moulder, 1991). The chlamydial lifecycle begins with an initial reversible electrostatic attachment of the EB to the host cell, followed by a secondary irreversible receptor-mediated binding during which the cytoskeleton is reorganised beneath the EB, culminating in internalisation and formation of an inclusion (Figure 2.1) (Betts *et al.*, 2009). Avoiding lysosomal fusion by subverting the endocytic pathway, the EB differentiates into the productive RB form, replicates by binary fission and de-differentiates back into the EB form, all inside the membrane-bound inclusion. At this point, progenies are released by extrusion and/or cytolysis to re-infect new cells or host (Hybiske and Stephens, 2008). An additional viable but non-cultivatable state, termed an aberrant body due to their appearance, has also been identified *in vitro*. Inducers of the aberrant phenotype include antibiotic treatment, interferon (IFN)-γ , amino acid starvation, iron deprivation, host cell differentiation state and co-infection with herpes simplex virus (HSV) (Hogan *et al.*, 2004; Wyrick, 2010). *In vitro*, aberrant bodies have been shown to lay dormant for up to 9 months and are capable of reverting to back to infectious EBs after the removal of the metabolic stress (Hogan *et al.*, 2004). Although this persistent phenotype has never been definitively proven to exist *in vivo* (Wyrick, 2010), it is thought to have links with infection re-emergence, chronic infection, and disease in humans (Richmond *et al.*, 1972; Hogan *et al.*, 2004).

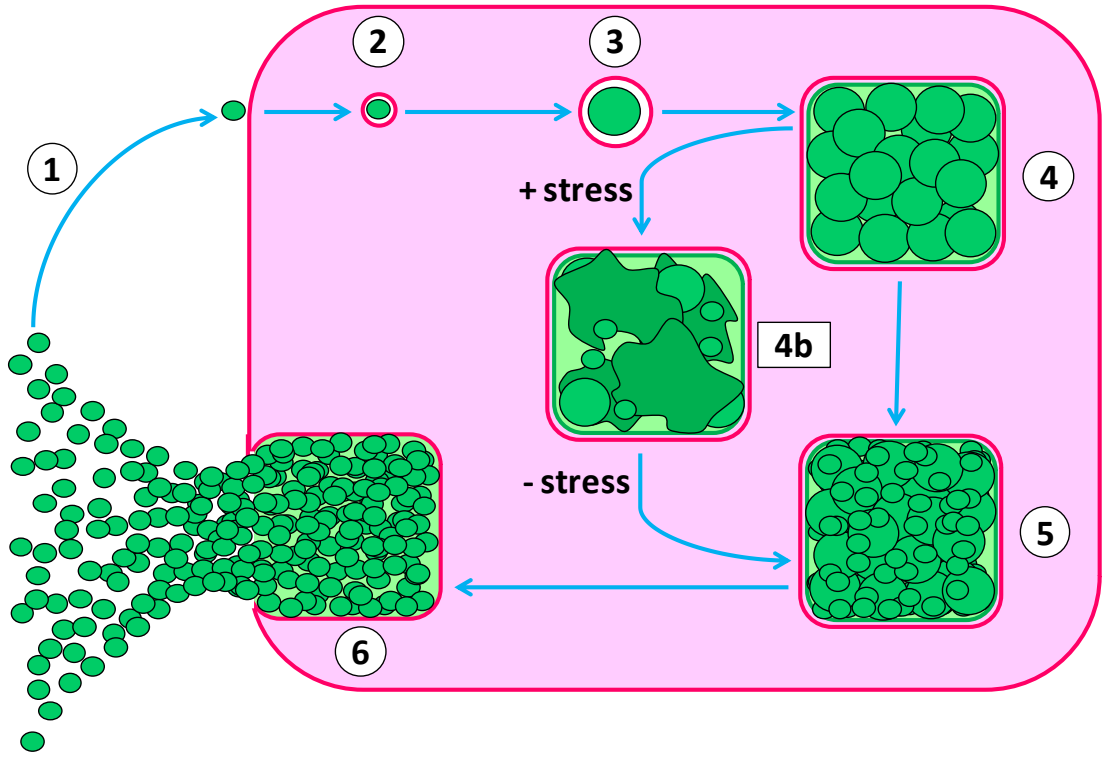


Figure 2.1: Simplified developmental cycle of *Chlamydia*.

(1) A chlamydial EB attaches to the host cell surface, (2) is internalised and forms an intracellular inclusion. (3) The EB differentiates into the divisible RB and (4) undergoes numerous rounds of replication. Following binary fission, (5) RBs begin to de-differentiate back into infectious EBs. (4b) Metabolic stress on the inclusion can induce a persistent phenotype characterised by the appearance of large aberrant chlamydial bodies. Following the alleviation of chlamydial growth stress, normal replication resumes. Replication is complete when infectious EB particles are released by cytolysis or (6) extrusion (Brunham and Rey-Ladino, 2005).

TRANSMISSION, PREVALENCE AND ASSOCIATED DISEASES

Chlamydia trachomatis

C. trachomatis (serovars D – K) is transmitted from person to person through unprotected sexual contact and is the most common cause of bacterial STI worldwide (WHO, 2009). Estimates from the World Health Organisation (WHO) report that *C. trachomatis* (serovars D to L) is responsible for 92 million of the 500 million new STIs annually. In Australia, notifications of sexually transmitted *C. trachomatis* account for 81% of all STIs and cases have increased from 14,045 in 1999 to 74,305 in 2010 (ABS, 2012). Prevalence of infection is 2-fold higher in adolescents (18 – 26 years) (Fenton *et al.*, 2001; Parish *et al.*, 2003; Franceschi *et al.*, 2007) and women are 2 – 3 times more likely than men to contract an infection (Gerbase *et al.*, 1998).

The social stigma attached to STIs also plays a contributory role in the spread of infection. Only 25% of symptomatic patients seek medical assistance (Liu *et al.*, 2002), the majority of which are reluctant to notify their spouse and admit to continuing to have sex despite the presence of symptoms (Liu *et al.*, 2002). *C. trachomatis* is Latin-derived from the term "to cloak", which ironically reflects the silent nature of the infection. Women have the highest incidence of asymptomatic infection, with 70 – 90% compared to 40 – 60% in men (Stamm and Holmes, 1990). As a result the majority of infections continue undiagnosed and untreated. Chronic chlamydial infection in women can cause urethritis, cervicitis, endometritis and salpingitis, the long term consequences of which include PID, ectopic pregnancy and tubal factor infertility (Patel *et al.*, 2008). Up to 40% of untreated infections in females develop PID, in which one in four cases result in infertility (WHO, 2007). This is thought to occur by the loss of cilia, which facilitate the movement of the oocyte, spermatozoa and tubular fluid, and by adhesions forming in/around the Fallopian tubes due to chronic inflammation (Lyons *et al.*, 2006; Hvid *et al.*, 2007; Kessler *et al.*, 2012). Women account for 80% of the total expense of treating chlamydial infections (Patel *et al.*, 2008), which cost in the order of $10 billion each year in the US alone (WHO, 2012). Neonates can also contract *C. trachomatis* as a result of an untreated maternal infection, with transmission occurring in 10 – 50% of deliveries (Smith and Taylor-Robinson, 1993). Chlamydial conjunctivitis causes up to 4000 infants to become blind each year and chlamydial pulmonary infections in early-life may contribute to the development of chronic respiratory tract diseases like asthma (Horvat *et al.*, 2007; Horvat *et al.*, 2010). The higher prevalence of infection and incidence of asymptomatic cases, the greater proportion of economic burden attributed to women and health risk to the unborn children, highlights the need for intervention particularly in females.

Chlamydia pneumoniae

Chlamydia pneumoniae is a respiratory tract pathogen transmitted from person to person in water droplets expelled by coughing or sneezing. Transmission is most common in areas of high population density, like schools, military bases and work places. Serological evidence suggests that 50% of people will have antibodies to *C. pneumoniae* by age 20, increasing to 80% by old age, indicating that the majority of

the population will contract an infection at one point in their lifetime (Grayston, 2000). Moreover, 75% of first infections occur in early life, which highlights the need for early intervention to prevent infection (Aldous *et al.*, 1992; Miyashita, 2006). *C. pneumoniae* infections are primarily asymptomatic, but in 30% of cases, can develop into a severe respiratory tract illness like pneumonia, of which *C. pneumoniae* is the third most common cause (Kuo *et al.*, 1995; Grayston, 2000). Re-infections are frequent and can survive for years despite antibiotic treatment (Hammerschlag *et al.*, 1992; Dean *et al.*, 1998; Grayston, 2000). Persistent infections can exacerbate chronic inflammatory lung diseases like asthma and COPD (Clementsen *et al.*, 2002), hypothesised to occur as a direct consequence of repeated unsuccessful attempts by the immune system to eradicate the infection. Factors contributing to a worsening disease state include the dysregulation of T helper type 2 (Th2)-immunity, excessive scar formation and narrowing and/or obstruction of the airways (von Hertzen, 1998). Prolonged chlamydial infections can generate a Th2-dominant response (Holland *et al.*, 1993; von Hertzen, 1998). This response does not facilitate infection resolution, but instead, the survival and dissemination of the pathogen throughout the body potentially using monocytic cells as a shuttle system (Gaydos, 2000; Grayston, 2000; Beagley *et al.*, 2009a). *C. pneumoniae* has been isolated from atherosclerotic plaques in coronary arteries (Kuo *et al.*, 1993), which through similar inflammatory mechanisms as mentioned above, could contribute to CVD by hardening and narrowing of arteries (Belland *et al.*, 2004). Self-reactive antibodies generated following a *C. pneumoniae* infection by a process of antigenic mimicry (Bachmaier and Penninger, 2005), have also been associated with other autoimmune diseases, such as Alzheimer's disease, MS and reactive arthritis. Therefore, due to the asymptomatic nature of infection there is an overwhelming need to prevent *Chlamydia* infection and minimise sequelae.

STRATEGIES FOR CONTROLLING *CHLAMYDIA*

The continual burden of *Chlamydia* and inability to eradicate infection from the community lies largely in the asymptomatic nature of the infection. There are currently two approaches towards controlling *Chlamydia*, (1) mass surveillance, detection and antibiotic treatment or (2) the development of an effective vaccine. The

mass surveillance and treatment strategy works by screening an entire healthy, generally high risk, population to identify and treat infected persons who were otherwise unaware of an active infection. Antibiotic invention strategies against *C. trachomatis* have proven a cost-effective method to reduce the economic burden associated with PID, even in demographics with low chlamydial infection prevalence (Howell *et al.*, 1998; Patel *et al.*, 2008). In these studies, *C. trachomatis* infections are primarily diagnosed by performing the nucleic acid amplification test (NAAT) and treated with a single dose of 1000mg of azithromycin (Dukers-Muijrers *et al.*, 2012). This screening and treatment process however has numerous short-comings when compared to widespread vaccination. The NAAT method of infection detection has continual problems with specificity and reproducibility, leading to misdiagnosis (Hadgu and Sternberg, 2009). Furthermore, there is an increasing body of evidence to suggest that the standard single-dose of azithromycin is ineffective against eradicating an infection (Drummond *et al.*, 2011; Dukers-Muijrers *et al.*, 2012), as 10 – 15% of women treated with antibiotics report a recurrent or persistent infection (Workowski *et al.*, 1993). In addition to the potential emergence of antibiotic resistance (Sandoz and Rockey, 2010), antibiotic intervention has be suggested to interfere with the development of the natural herd immunity created by widespread infection (Su *et al.*, 1999). Case detection and antibiotic treatment strategies employed in Sweden, Norway, Finland, Canada, US and Australia in the late 1980's all failed to stem the rise in infection prevalence long-term (Brunham and Rekart, 2008). Moreover, as a result of these control programs, re-infection rates actually increased in some countries by much as 5% per year (Brunham *et al.*, 2005). Dubbed the "arrested immunity hypothesis", it was suggested that by terminating an infection prematurely with antibiotics prevented the development of immunity against re-infection, leading to an increased prevalence in the community (Brunham *et al.*, 1996; Bailey *et al.*, 1999; Brunham *et al.*, 2005). It was also recently discovered that the current clinical practice of testing patient samples 3 weeks following treatment with azithromycin, for a test-of-cure, was found to miss over 40% of unresolved infections (Dukers-Muijrers *et al.*, 2012).

It is conceivable that mass screening and treatment strategy would be equally if not more ineffective against the control of *C. pneumoniae*. *C. pneumoniae* infections are

extremely difficult to diagnose due to the low organism titres in throat and sputum samples (Grayston, 2000). Hence detection of *C. pneumoniae* is often reliant on the unapproved serological the microimmunofluorescence (MIF) test (Dowell *et al.*, 2001; Kumar and Hammerschlag, 2007), which is also unable to accurately differentiate between past and present infections (Villegas *et al.*, 2010). *C. pneumonia* infection have also been reported to persist for years despite frequent and extended course antibiotics (Hammerschlag *et al.*, 1992).

Therefore, the current strategy of detection and treatment is not only ineffective at controlling the prevalence of infection (Brunham *et al.*, 2005; Brunham and Rekart, 2008), but could in fact be contributing towards the uncontrolled spread of infection (Hadgu and Sternberg, 2009). As a result an efficacious vaccine is recognised by both the WHO and Centres for Disease Control (CDC) to have the greatest potential to impact on infection and disease prevalence (Brunham *et al.*, 2005; Gray *et al.*, 2009).

CONTROLLING *CHLAMYDIA* THROUGH VACCINATION

The ultimate goal of a chlamydial vaccine is to reduce the prevalence of disease (eg. infertility), to which all healthcare expenditure is linked. A chlamydial vaccine would ideally target both males and females for the greatest impact on infection and disease prevalence (Gray *et al.*, 2009). However, there are limitations to the type of adaptive immune responses that can be generated in the male reproductive tract, which complicates the development of a vaccine that elicits protection against infection in both males and females. The testes are considered an "immune-privileged" site, which means they can tolerate low levels of foreign antigen without eliciting an immune response (Fijak and Meinhardt, 2006). As spermatozoa mature during adolescences following the development of self-tolerance (Tung *et al.*, 1971; Tung *et al.*, 1981), suppression of inflammation in the testes is crucial to prevent autoimmunity to sperm. This is a mechanism of self-preservation to prevent infertility by autoimmunity. The blood-testis barrier is the primary impediment to the generation of auto-reactive antibodies against spermatozoa (Fijak and Meinhardt, 2006). A pro-inflammatory cell-mediated response, required for optimal protection against a chlamydial infection (Morrison and Caldwell, 2002), has been shown to

disrupt the blood-testis barrier and lead to autoimmune orchitis and infertility (Matsuzaki *et al.*, 1997; Itoh *et al.*, 1998). Furthermore, as cell-mediated immunity generated following exposure to *C. pneumoniae* can respond to *C. trachomatis* antigens (Telyatnikova and Hill Gaston, 2006), a vaccine against a *Chlamydia* respiratory tract infection may also need to be restricted to females to prevent any potential cross-reaction and inflammation caused by the cell-mediated response in the male genital tract. Some studies suggest males should be a priority (Patterson and Rank, 1996) and that a chlamydial vaccine that solely induces immunoglobulin A (IgA) could provide partial protection against infection without causing autoimmunity (Hickey *et al.*, 2004). However, the predominant focus of chlamydial vaccine design has been towards a female vaccine, as most of the severe sequelae occurs in woman (Patel *et al.*, 2008). A positive impact on infection and disease can be achieved if a female vaccine meets one of the two following scenarios.

(1) A vaccine completely prevents an infection from becoming established. Thus, transmission of infection cannot occur or diseases develop if the infectious *Chlamydia* are neutralised before undergoing even a single round of replication. This is known as "sterilising immunity", where a vaccine elicits completely immunity to infection and disease following exposure, without the possibility of transmission. Mathematical modelling suggests a vaccine eliciting sterilising immunity against *C. trachomatis* could potentially eradicate all infection from the human population within a 20 year time frame (Gray *et al.*, 2009). Only a prior infection has proven successful in animal models to provide complete, albeit transient, sterilising immunity (Barron *et al.*, 1984; Rank *et al.*, 1988a; Ramsey *et al.*, 1989; Patterson and Rank, 1996). The protection and longevity of immunity acquired following a natural infection, is predominantly dependent on the recruitment and maintenance of T cells at the site of infection (Ramsey *et al.*, 1989; Igietseme and Rank, 1991; Su *et al.*, 1997; Igietseme *et al.*, 2000; He *et al.*, 2005). Therefore, a vaccine that aims to elicit complete and long-lived sterilising immunity against *Chlamydia* must retain T cell responses in the mucosal tissues. The recruitment of T cells into the genital tract is possible following vaccination (Marks *et al.*, 2011a). The induction of lymphoid aggregates and long-lived tissue-resident memory T cells (T_{RM}) in the cervico-vagina has also been described in mice and humans following viral and bacterial genital

tract infections (Kiviat *et al.*, 1990; Mackay *et al.*, 2012). However, a continual presence of activated T cells could increase the incidence of adverse pregnancy outcomes (Maruyama *et al.*, 1992) and susceptibility to other more life-threatening STIs like human immunodeficiency virus (HIV) (Levine *et al.*, 1998). Maintaining protective T cells in the genital tract is not only undesirable, but also unlikely. In the absence of continual cytokine and chemokine stimulation, most T cells (except T_{RM}) preferentially re-circulate back into the periphery, which would alleviate sterilising immunity against *Chlamydia* (Hawkins *et al.*, 2000). Moreover, mice have been shown to possess IL-10-secreting dendritic cells (DCs) in the lower genital tract that suppress protective T cell immunity (Marks *et al.*, 2011b). Perhaps the greatest barrier against retention of T cells in the genital tract is the monthly tissue re-modelling and anti-inflammatory hormonal fluctuations that occur in the female genital tract over the estrous cycle. T_{RM} have been found lodged in the vagina and can persist in the skin beyond 1 year following treatment with a non-specific inflammatory stimulus, however it is currently unclear what effect sex hormones will have on their ability to populate the epithelium. Although, complete protection is most desirable, induction of sterilising immunity is becoming increasingly unlikely (Beagley *et al.*, 2009b). No vaccine to date has been capable of inducing sterilising immunity and due to the nature of the pathogen and the physiology of the genital tract it infects; such a vaccine is unlikely to emerge in the near future.

(2) If the induction of sterilising immunity is not possible, a vaccine that prevents the development of pathology by stopping the infection from ascending to the upper reproductive tract and reduced the chance of further sexual transmission by lowering the duration and/or quantity of *Chlamydia* shed into the genital tract may also be beneficial (Gray *et al.*, 2009). Figure 2.2 illustrates the mechanism in which a non-sterilising vaccine can enhance herd immunity and potentially eradicate infection and disease from the human population. The chlamydial load shed during a natural course of infection (red line) is far greater than the critical bacterial load required to infect a naive individual (black dotted line), thus the infection is transmitted to the next susceptible host. A vaccine can raise the infectious threshold (green dotted line) to a level near the maximum chlamydial load shed during a natural course of infection (red line), which reduces the duration a vaccinated individual is susceptible

to contracting an infection. Vaccination can also reduce the chlamydial load shed over the course of an infection (green line), again minimising the duration that a susceptible host can be infected (black dotted line). Transmission can be prevented entirely between two vaccinated individuals, as the lowered chlamydial load shed (green line) does not surpass the elevated threshold (green dotted line) necessary for infection transmission. Therefore, implementing a vaccine conveying even partial protection i.e. increasing infectious threshold, reducing pathology and chlamydial shedding could also have a positive effect on *Chlamydia*-associated sequelae (Gray *et al.*, 2009).

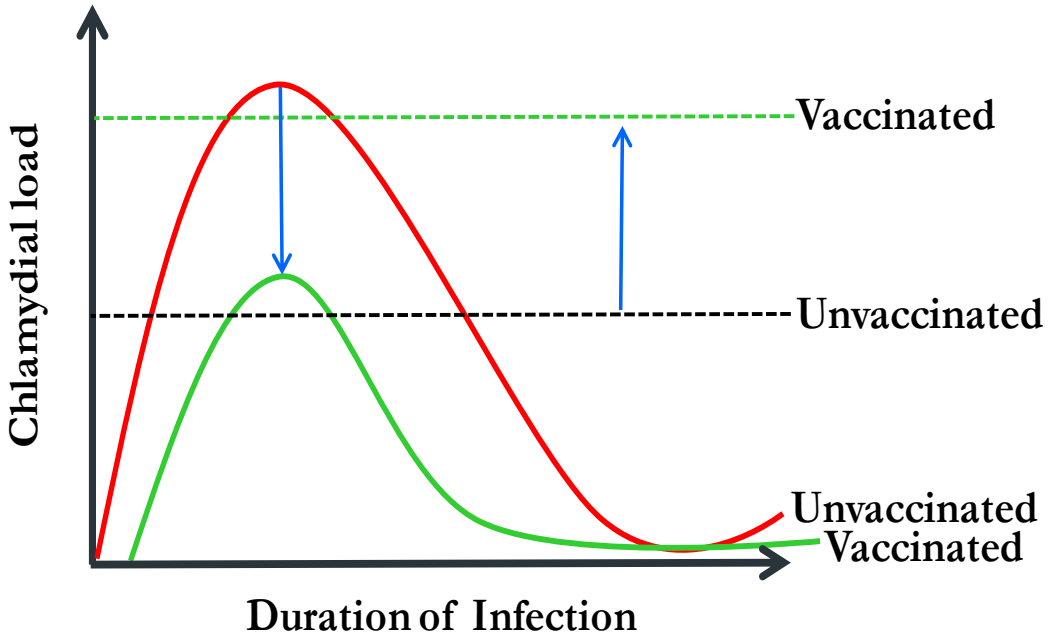


Figure 2.2: Mechanisms of herd immunity induced by non-sterilising vaccines.
The solid lines represent the chlamydial load shed during an active infection (vaccinated and unvaccinated), while the dotted lines signify the chlamydial load required to establish an infection in a new host (vaccinated and unvaccinated) (Gray et al., 2009). If the infection threshold is raised following vaccination (green dotted line), the vaccinee is susceptible to contracting an infection, but only from an unvaccinated person at the peak of their bacterial shedding (red solid line). If the peak of chlamydial load can be reduced following vaccination (green solid line), an unvaccinated person is susceptible to contract an infection, but only from the infected vaccinee at the peak of their bacterial shedding (black dotted line). However, between two vaccinees, the peak of chlamydial load shed at the peak of their infection does not meet the minimum infectious threshold for transmission to occur.

HUMAN IMMUNE RESPONSE TOWARDS CHLAMYDIA

To design an effective human vaccine against *Chlamydia*, it is important to understand human infection kinetics. This means identifying the immune mediators influencing infection resolution and development of pathology. Unfortunately, ethical considerations are the major challenge impeding the study of untreated chlamydial infections in humans, as once an infection is detected it must be treated promptly with antibiotics. However, an intimate knowledge of human infection will help develop more accurate animal models, more capable of predicting a vaccines potential to prevent infection transmission and reduce disease in a human population.

C. trachomatis

Factors involved in the resolution of a human infection and development of pathology are still largely unknown. Women with a prior history of chlamydial infection(s) generally resolve an infection faster than those without a previous infection (Geisler et al., 2008), indicating the presence of partial immunity after repeat infections. Despite both T and B cells being recruited to the lower genital tract following infection (Mittal *et al.*, 2004; Ficarra *et al.*, 2008), the cell-mediated response appears more important than antibodies in the context of a human genital tract infection. Polymorphisms in antigen-presenting molecules, human leukocyte antigen (HLA) class I and II, were associated with higher incidences of chlamydial infections (Geisler *et al.*, 2004), suggesting that T cell priming is important in human to prevent and resolve infections. HIV-positive patients, with a reduction in cluster of differentiation (CD) 4^+ T cells, were also determined to be at a greater risk of developing PID (Kimani *et al.*, 1996), indicating a role for $CD4^+$ T cells in preventing infection ascension and pathology in humans. Levels of IFNγ were also greater in cervical/vaginal secretions of women and non-human primates with recurrent infection (Van Voorhis *et al.*, 1997a; Agrawal *et al.*, 2007), which demonstrates that T helper type 1 (Th1) cytokines increase upon repeated infections and are associated with increased immunity and reduced disease (Debattista *et al.*, 2002; Kinnunen *et al.*, 2002). Systemic and mucosal antibodies (IgA and IgG) can be detected following a natural infection in women (Mittal *et al.*, 1996; Pate *et al.*, 2001; Ghaem-Maghami *et al.*, 2003; Agrawal *et al.*, 2007), although there is no

association between IgG levels and protective immunity in non-human primates (Wolner-Hanssen *et al.*, 1991). There is however some negative association between serum antibody levels and severity of disease following *C. trachomatis* infections in humans (El Hakim *et al.*, 2010), particular against chlamydial antigens which mimic certain human proteins like heat-shock protein (HSP) 60 (Bachmaier and Penninger, 2005). Acute inflammation, characterised by an influx of polymorphonuclear leukocytes (PMN), occurs in humans and non-human primates following infection (Ripa *et al.*, 1979; Kiviat *et al.*, 1990; Molestina *et al.*, 1999; Geisler *et al.*, 2005; Ficarra *et al.*, 2008; Patel *et al.*, 2010) and has strong associations the development of disease (Wiesenfeld *et al.*, 2002; Patel *et al.*, 2010). Cervical cells isolated from *Chlamydia*-positive fertile and infertile women secreted IFNγ and IL-12 or IL-1β, IL-4, IL-6, IL-8 and IL-10 when stimulated with *C. trachomatis*, respectively (Agrawal *et al.*, 2009). This suggests innate- (IL-1β, IL-6 and IL-8) and Th2-related (IL-4 and IL-10) cytokines contribute towards disease in humans following infection, while type-1 cytokines (IFNγ and IL-12) mediate the resolution of an active genital tract infection.

C. pneumoniae

The immunological information regarding *C. pneumoniae* infections in humans is predominantly based on serological analysis due to the invasiveness of lung sample collection methods. Despite the limited amount the human data available, the main factors implicated in mediating immunity appear similar between the two chlamydial species. The incidence of *C. pneumoniae* infections in HIV-positive patients is inversely proportion to $CD4^+$ T cell counts (Tositti *et al.*, 2005). Increased levels of IFNγ-secreting $CD4^+$ T cells can be detected in peripheral blood mononuclear cells (PBMCs) following a *C. pneumoniae* infection in humans, which also coincided with the infection resolution (Halme *et al.*, 2000; Bunk *et al.*, 2010). Antibodies to antigens like HSP60 also appear to have a negative association with severity of disease following *C. pneumoniae* infections in humans (Molestina *et al.*, 2002; Hoshida *et al.*, 2005; Mukhopadhyay *et al.*, 2006).

Overall, protection against infection and disease following a *Chlamydia* infection in humans requires a dominant cell-mediated response. *Chlamydia* infection research in

humans has obvious technical and ethical limitations (Geisler, 2010), thus progress towards a human chlamydial vaccine is largely reliant on animal models.

THE MOUSE AS A MODEL OF CHLAMYDIAL INFECTION

An animal model with the capacity to mimic the disease process elicited by *Chlamydia*, as it occurs naturally in the human host, not only provides important information on host/pathogen interactions but also an ethical means to test intervention strategies like vaccines. It is estimated that over half of highly cited animal research does not translate to the level of a human randomised trial due to inconsistencies between the model and humans (Ioannidis, 2005; Hackam and Redelmeier, 2006). Therefore, utilising an animal model that accurately reflects the many important aspects of human infection and disease is vital for the successful development of a vaccine for humans. Non-human primates (Van Voorhis *et al.*, 1997b), pigs (Schautteet and Vanrompay, 2011) and guinea pigs (Rank *et al.*, 2003) are the most desirable models for chlamydial infection research, as these best replicate human anatomy/physiology, infection, immunity and disease. These models however have numerous practical disadvantages that have restricted their widespread use. In contrast, mice have the benefits of size and ease of handling, relatively small financial cost and a plentiful supply of mouse immune reagents. Most importantly, mice also accurately represent most aspects of acute infections in humans (Morrison and Caldwell, 2002), making the mouse the experimental model of choice.

Host-specific tropisms

Two murine infection models using a variety of mouse backgrounds are in use today, one utilising either human pathogen *C. trachomatis* or *C. pneumoniae* the other the mouse-adapted pathogen *C. muridarum*. Each chlamydial species displays distinct host-specific tropisms, which have co-evolved to support optimal bacterial growth within a specific tissue of a certain host. Human *Chlamydia* species (*C. trachomatis* or *C. pneumoniae*) share more host-specific tropisms in common than non-human chlamydial species (*C. muridarum* or *C. caviae*) (Mital and Hackstadt, 2011). Divergence does however exist between human pathogens, particularly in regards to tissue-specificity (Moelleken and Hegemann, 2008). Tropisms are immensely

important to consider when choosing an animal model as these can include critical bacterial compensatory responses to their respective host's immune defences (Ito *et al.*, 1990; Beatty *et al.*, 1994; Caldwell *et al.*, 2003; Nelson *et al.*, 2005a; Shah *et al.*, 2005a; Coers *et al.*, 2008; Coers *et al.*, 2009; Ramsey *et al.*, 2009; Mital and Hackstadt, 2011). For example, IFNγ stimulates numerous antimicrobial actions (e.g. indoleamine 2,3-dioxygenase (IDO), inducible nitric oxide synthase (iNOS), iron deprivation and p47 guanosine-5'-triphosphate (GTP)ases, expression of which differs based on both the host species (human or mouse) and tissue (epithelial, fibroblast, macrophage/monocyte) (Roshick *et al.*, 2006). In human cells, the major protective mechanism against *C. trachomatis* is IDO-catalysed tryptophan degradation, which starves the auxotrophic chlamydial inclusion of the essential amino acid tryptophan (Beatty *et al.*, 1994). The validity of this mechanism has been supported *in vitro* by using tryptophan supplementation to restore *C. trachomatis* and *C. muridarum* replication in IFNγ pre-treated human cell lines (Nelson *et al.*, 2005a). The human-adapted *C. trachomatis* has evolved an immune evasion strategy within a highly variable genomic plasticity zone (Fehlner-Gardiner *et al.*, 2002). The *C. trachomatis* genital tract serovars D – K encode a functional tryptophan synthase (*trp*) that enables them to utilise extracellular indole, produced by commensal bacteria, to support chlamydial replication by circumventing IFNγ-mediated protection in humans (Caldwell *et al.*, 2003). The mouse-derived *C. muridarum* does not have the capacity to escape this protective effect, lacking vital *trp* genes (Caldwell *et al.*, 2003), hence the murine pathogen is more susceptible to IFNγ-induced IDO defence mechanism in human cell lines and cannot be rescued with the addition of indole (Nelson *et al.*, 2005a). A similar host tropism can be seen in murine cells in relation to the natural human pathogen *C. trachomatis*. Intravaginal (IVag) inoculation of mice with *C. trachomatis* in most cases results in a mild, self-limiting, lower reproductive tract infection, with minimal ascension to upper reproductive tract (Ito *et al.*, 1990). Some studies employ direct intrabursal inoculation to elicit the upper reproductive tract symptoms although this does not simulate the natural route of infection. Overall, the failure of *C. trachomatis* to cause a significant infection in the mouse suggests the major antimicrobial action induced by IFNγ in mice is independent of IDO (Nelson *et al.*, 2005a) and is more than likely a mechanism that *C. trachomatis* cannot avoid. Conversely, challenge of mice with

C. muridarum causes ascending infection and severe upper reproductive tract associated sequelae (hydrosalpinx, tubal occlusion and infertility) (Shah *et al.*, 2005a), similar disease spectrum to that seen following *C. trachomatis* genital tract infection in humans. This suggests that *C. muridarum* may also encode an IFNγ immune evasion strategy, which differs to *C. trachomatis*, but is capable of overcoming IFNγ-mediated protection in the mouse. IFNγ induced defence in mice is considerably more complicated and it is yet to be determined whether IFNγ has a direct inhibitory effect, as seen for *C. trachomatis* in human cells, or if protection is mediated by stimulating a particular T cell population (Cotter *et al.*, 1997a). The complexity is evident in a study by Nelson *et al.*, using IFNγ$^{-/-}$, IDO$^{-/-}$ and iNOS$^{-/-}$ mice, which were challenged with *C. trachomatis*. Only the IFNγ-deficient animals showed more severe infection when compared to wild type (WT) animals. This indicates that eradication of *C. trachomatis* in the mouse is independent of IDO, the principle mechanism of protection against *C. trachomatis* in human cells. Interestingly, when the same knockout (KO) strains were challenged with *C. muridarum*, contradictory to a number of previous reports (Rank *et al.*, 1992; Cotter *et al.*, 1997b), there was no difference in infection magnitude or duration signifying that protection in mice against *C. muridarum* is independent of IFNγ, IDO and iNOS. It is worth noting that conflicting studies used alternate strains of *C. muridarum* (Weiss verses Nigg), previously shown to have largely different virulence phenotypes (Ramsey *et al.*, 2009), which may imply that even within strains of *Chlamydia* both pathogen susceptibility and host response to IFNγ may vary considerably. Alternatively, there is another as yet undiscovered mechanism in the mouse capable of conferring protection against infection, but masked by the profound effects of IFNs. Looking more closely at protein expression in IFNγ-stimulated mouse cells Nelson *et al.*, (2005a) implicated a family of p47 immunity-related GTPases (IRGs) in the inhibition of *C. trachomatis* growth in mice. The authors suggested that IRGs may reduce lipid trafficking to the chlamydial inclusion and concluded that *C. muridarum* may inactivate host-cell IRGs as their immune evasion strategy. The list of factors in mice contributing to resistance to *C. trachomatis* infection has been refined to IRG genes *irgm*1, *irgm*3 and *irgb*10 (Coers *et al.*, 2008), and *C. muridarum* escape, to cytotoxins encoded in the plasticity zone inactivating IRG effector proteins (Nelson *et al.*, 2005a). Recent studies have sought

to confirm the role of IRGs in mice during a *C. trachomatis* (IRG-sensitive) infection *in vivo*, by utilising *irgm*1/*irgm*3 double-deficient mice (Coers *et al.*, 2011). *Irgm*1/*irgm*3$^{-/-}$ mice initially displayed an increase in bacterial burden, but cleared an infection as effectively as the WT animals. Therefore, the control of an infection in its early stages requires the innate effector mechanisms driven by *irgm*1 and *irgm*3. In the absence of *irgm*1 and *irgm*3 however, a compensatory adaptive immune response was responsible for the normal resolution of an active infection. While there are many questions that still remain unanswered regarding mechanisms of IFNγ-mediated protection, there are distinct differences in not only the human and mouse-adapted *Chlamydia* but also in the respective host's responses induced by IFNγ, which compromise the efficacy of the current mouse models. Without the availability of transgenic humanised mice, replacing the IRG-mediated resistance system (mouse) with the IDO-mediated resistance system (humans) (Coers *et al.*, 2009), it would appear rational to use the mouse with its co-evolved and natural pathogen *C. muridarum* to replicate human *Chlamydia* infections for vaccine trials.

Genital tract infection model

Progesterone-primed female mice can be infected with *C. muridarum* via the vaginal vault (Ramsey *et al.*, 2009), the natural route of a *C. trachomatis* infection in humans. An IVag infection in mice leads to the colonisation of the vagina and a period of shedding infectious chlamydial organisms for a period of 14 – 50 days (Cochrane *et al.*, 2010). Shortly after the establishment of the infection in the lower reproductive tract, the infection ascends to colonise the upper reproductive tract (cervix, uterine horns and oviducts) (Carey *et al.*, 2009). This is followed by the development of hydrosalpinx, which is an accumulation of clear serous fluid in the oviduct (Carey *et al.*, 2009), the mouse equivalent to the human Fallopian tubes. Hydrosalpinx is characteristic of the scarring associated with tubal factor infertility in humans and the swelling size of the oviduct is directly proportional to the severity of pathology and extent of the blockage (Shah *et al.*, 2005b; Imtiaz *et al.*, 2006) These blockages are thought to occur by similar mechanisms to humans (Kessler *et al.*, 2012), by scarring and adhesion of the oviducts and the disruption of the cilia function/contractile activity responsible for clearing oviduct secretions (Shibahara *et al.*, 2001; Dixon *et al.*, 2010). The major disadvantage of the mouse genital tract

infection model is that mice require an initial pre-treatment with progesterone to enhance susceptibility to an IVag infection by synchronising animals in diestrus (Tuffrey and Taylor-Robinson, 1981), which can alter the immunological balance of the mice to favour Th2 cytokine production (Kita *et al.*, 1989; White *et al.*, 1997).

Respiratory tract infection model

Mice can be infected by inhalation of *C. muridarum* (Ramsey *et al.*, 2009), which is the natural route of a *C. pneumoniae* infection in humans. Animals infected intranasally with *C. muridarum* undergo a stage of dramatic weight loss known as cachexia. Cachexia is indicator of chronic inflammation and development of pneumonia, which effects energy expenditure and eating behaviour resulting in weight loss (van Heeckeren *et al.*, 2000). *C. muridarum* infected bone marrow-derived DC (BMDCs) can also polarise naive $CD4^+$ T cells during antigen presentation to adopt a Th2 phenotype and produce IL-4, IL-5 and IL-13 (Kaiko *et al.*, 2008). This may enhance IgE and mucus production, eosinophil infiltration and mediate the thickening and fibrotic scarring of the airways associated with COPD, allergic sensitisation and exacerbation of asthma (Horvat *et al.*, 2007; Kaiko *et al.*, 2008; Horvat *et al.*, 2010). Similar to *C. pneumonia* infection in humans, *C. muridarum* can also disseminate throughout the mouse following an IN infection (Jupelli *et al.*, 2008). Although the specific mechanisms are still undefined in the mouse, BMDCs can be infected with *C. muridarum in vitro* and then transmit an infection to recipient mice following their adoptive-transfer (Rey-Ladino *et al.*, 2007). *C. muridarum* is also able to colonise and inflame cardiac tissue following IN infection, a contributory risk factor in the development of CVD (Fan *et al.*, 1999). Autoimmune diseases in humans attributed to antigenic mimicry have also been reported to some extent in the mouse model. A systemic infection of mice with *Chlamydia* increased the severity of experimental allergic encephalitis (EAE) (Du *et al.*, 2002), which is largely considered the animal model of MS. Intra-articular injection of *C. muridarum* into the knee joint after an initial sensitisation with chlamydial antigens, causes the histopathological changes associated with arthritis. However, the role cross-reactive antibodies play in pathogenesis in the mouse is still unclear (Hough and Rank, 1988; Rank *et al.*, 1988b; Du *et al.*, 2002). While the mouse model accurately replicates the many diseases associated with a *C.*

trachomatis and *C. pneumoniae* infection in humans, it is also important that the mechanisms of pathogenesis are similar to that which occurs in humans to accurately assess potential vaccines.

THE MOUSE AS A MODEL OF CHLAMYDIAL PATHOGENESIS

One significant difference between mice and humans is that infections in mice are self-limiting and pathology often develops after a single challenge. Contrastingly, humans can develop long-term chronic infections (Dean *et al.*, 1998) and the risk of developing pathology increases after repeated infections (Kimani *et al.*, 1996; Hillis *et al.*, 1997; Bakken *et al.*, 2007). Currently, two hypothesises have been proposed to explain the immune-mediated pathogenesis associated with chlamydial infection, the cellular- and immunological-paradigms (Stephens, 2003; Brunham and Rekart, 2008; Darville and Hiltke, 2010).

Cellular paradigm

The cellular-paradigm (Figure 2.3), suggests that pathogenesis is initiated and sustained by the pro-inflammatory cytokines/chemokines secreted by *Chlamydia*-infected epithelial cells, which in an ongoing chronic infection promote cellular proliferation, tissue remodelling and scarring. Local production of pro-inflammatory cytokines have been shown to be a key initiator of direct tissue destruction, even in the absence of leukocytes (Hvid *et al.*, 2007). However, these pathological effects of infection are also likely to be exacerbated further following the recruitment and infiltration of tissue-damaging innate and adaptive immune cell populations (Lee *et al.*, 2010b).

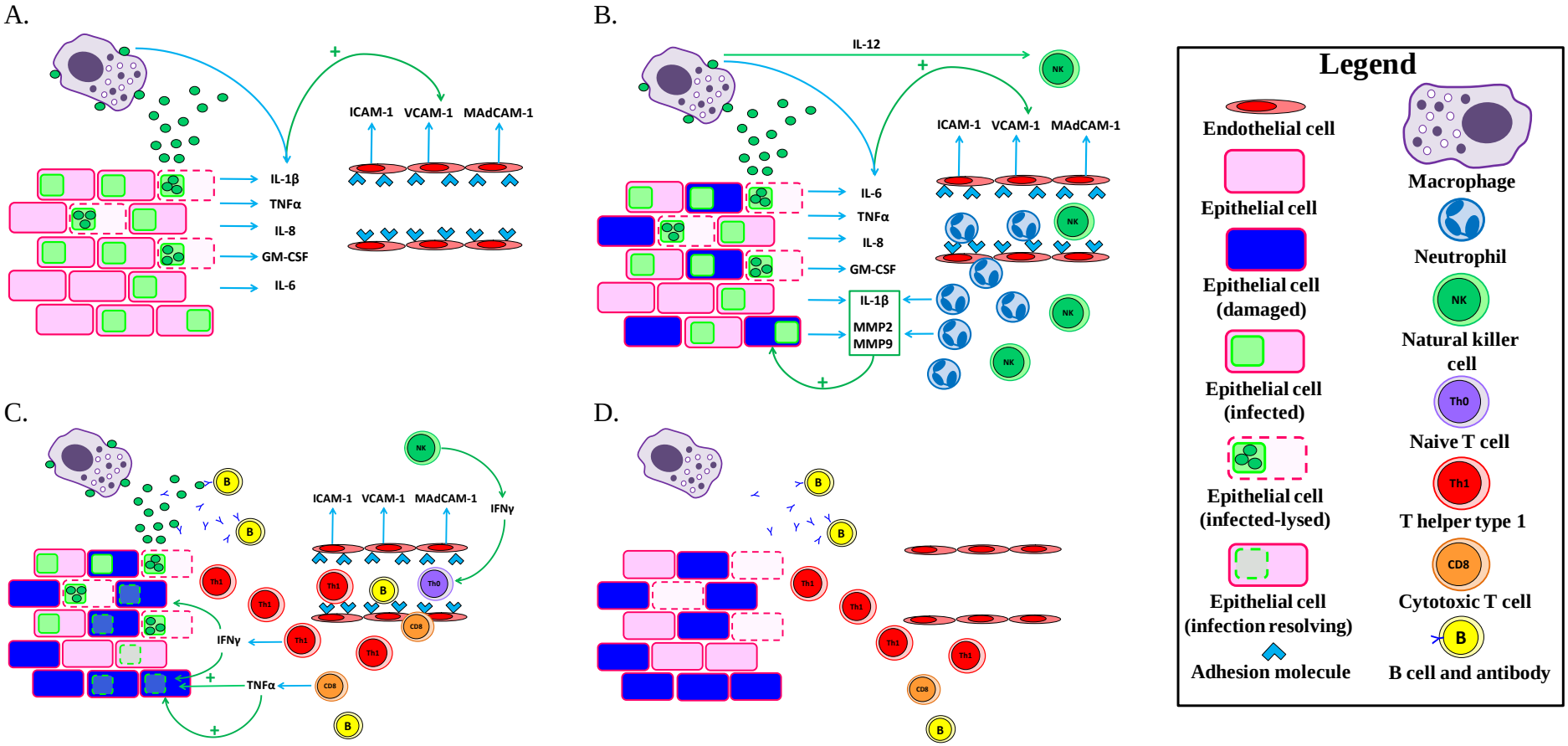


Figure 2.3: Illustration of cellular paradigm of pathogenesis.

(A) The epithelium and local innate populations like macrophages secrete pro-inflammatory cytokines and chemokines (including IL-1β, tumour necrosis factor (TNF)-α, IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF)) in response to an infection. This increases expression of adhesion molecules (intercellular adhesion molecule (ICAM)-1 and mucosal addressin cell adhesion molecule (MAdCAM)-1/vascular cell adhesion molecule (VCAM)-1) on the endothelium. (B) Epithelial cells together with the newly recruited neutrophils secrete matrix metalloproteinases (MMPs) and more pro-inflammatory cytokines, which promote extracellular matrix deposition and damage to the epithelia. (C) The

adaptive immune response consisting of $CD4^+$, $CD8^+$ and B cells are recruited and polarised towards Th1 response by IFNγ-secreting natural killer (NK) cells. (D) Following the resolution of infection, inflammation subsides, leaving tissue damage (Darville and Hiltke, 2010).

Immunological paradigm

The immunological-paradigm (Figure 2.4) involves two aspects, a delayed-type hypersensitivity (DTH) reaction and/or autoimmune cross-reactivity (molecular mimicry) (Bachmaier and Penninger, 2005; Swanborg *et al.*, 2006). Support for this paradigm comes mostly from the primate (Van Voorhis *et al.*, 1997b) and guinea pig (Rank *et al.*, 1995) models, more akin to human anatomy and physiology. It shows that pathology worsens upon repeat infections and associates with the exponential increase in the proportion of *Chlamydia*-specific $CD4^+$ and $CD8^+$ cells, compared to innate leukocytes, recruited to the site of infection. Whilst T cells are known to mediate infection resolution, they can also promote immunopathology and persistence of infection, leading to tissue damage (Holland *et al.*, 1996; Van Voorhis *et al.*, 1997a; Wang *et al.*, 1999; Yang *et al.*, 1999; Rank *et al.*, 2000). In addition, auto-reactive T cells and antibodies directed toward chlamydial HSP60, which shares approximately 50% homology with human HSP60, could also increase upon recurrent infection. These also have the potential to cause severe tissue damage (Peeling *et al.*, 1997), but these claims are yet to be completely substantiated (Ness *et al.*, 2008).

Despite mice and human kinetics of pathogenesis appearing to support opposing paradigms, both hypotheses are not mutually exclusive and studies have implicated the existence of either paradigms in mice and humans (Hvid *et al.*, 2007; Lu *et al.*, 2011). The key point is however that chlamydial pathogenesis occurs as a result of the host immune response (innate or adaptive) and not the direct effect of the bacteria, which complicates vaccine design.

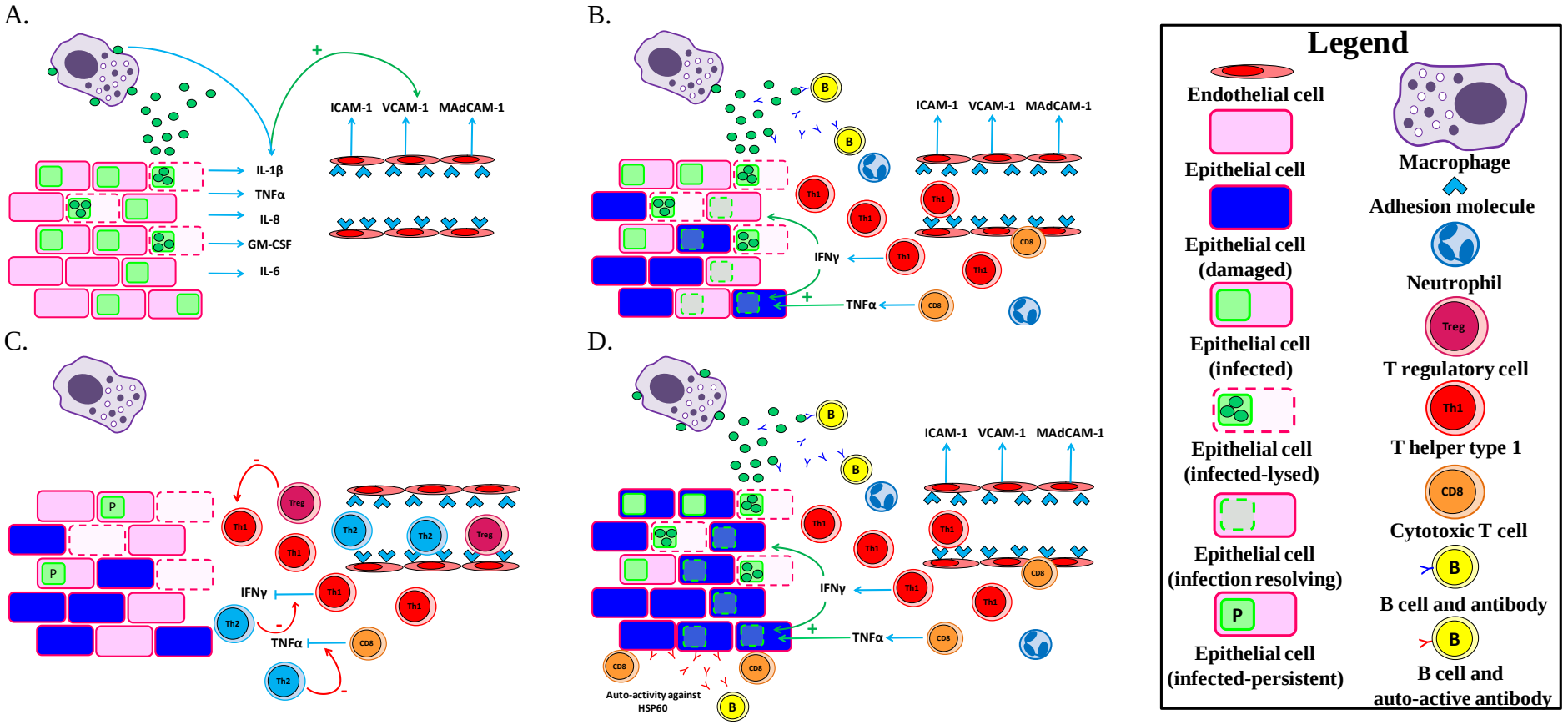


Figure 2.4: Illustration of immunological paradigm of pathogenesis.

(A) Following re-infection, the epithelium and local innate populations secrete pro-inflammatory cytokines and chemokines, which increase expression of adhesion molecules on the endothelium. (B) The resulting influx of cells is dominated by pro-inflammatory $CD4^+$ and $CD8^+$ adaptive immune cells, which facilitate the resolution of infection by secretion of pro-inflammatory cytokines and potentially contribute towards immunopathology. (C) An anti-inflammatory response driven by Th2 and inducible T regulatory cells (T_{reg}) may suppress tissue-damaging inflammation, but also promote persistence of any residual uncleared infection. (D) A recurrent infection or reactivation of a persistent infection may cause further tissue damage

through the expansion of an even greater adaptive immune response, which may include HSP60-specific auto-reactive B and T cells that damage the epithelia directly.

THE MOUSE AS A MODEL OF PROTECTIVE IMMUNITY

To develop an effective vaccine, it is important to understand how each aspect of the immune response protects against a chlamydial infection or contributes towards pathology. This section will discuss protective immunity in the *C. muridarum* model, in the context of both reproductive and respiratory tract infections. Reference to the *C. trachomatis* and *C. pneumoniae* mouse models will also be included, but only for key studies or when no information on *C. muridarum* is available. Similarities and differences between the mouse and human models will also be included where appropriate.

The innate immune response

The innate immune response is the first line of defence against an infection. The primary function of the innate response during a chlamydial infection is to control and contain an infection, but also recruit and activate the adaptive immune response. The innate response, by comparison to the adaptive response, is inefficient at clearing a chlamydial infection (Rank *et al.*, 1985; Cotter *et al.*, 1997b; Hawkins *et al.*, 2002) and is commonly associated with the exacerbation of disease.

Pattern recognition receptors (PRRs)

The innate response is triggered following recognition of conserved microbial structures called pathogen-associated molecular patterns (PAMPs) or by nuclear/cytosolic material released during tissue injury known as damage-associated molecular patterns (DAMPs). Toll-like receptors (TLRs), the nucleotide-binding, oligomerisation domain (NOD)-like receptors (NLRs), the retinoic acid-inducible gene-like receptors (RLRs) and the C-type lectin receptors (CLRs) are the major groups of PRRs, responsible for detecting PAMPs and DAMPs. These receptors are expressed predominantly by innate immune effector cells, such as macrophages, neutrophils and DCs that are abundant in the mucosa, as well as both epithelial and endothelial cells (Kawai and Akira, 2005; Joyee and Yang, 2008). *C. muridarum* interacts with a number of surface (TLR2, 3 and 4) and cytosolic PRRs (TLR9 and Nod1) (Darville *et al.*, 2003; Welter-Stahl *et al.*, 2006; Joyee and Yang, 2008; Ouburg *et al.*, 2009; Derbigny *et al.*, 2010; He *et al.*, 2011; Derbigny *et al.*, 2012),

although only chlamydial lipopolysaccharide (LPS) and HSP60 have been definitively identified as PAMPs (Joyee and Yang, 2008). Interestingly, it is the subtle differences in innate cytokine responses influenced by PRR ligation between mouse strains that control susceptibility to infection and pathogenesis (Darville *et al.*, 2001; Watanabe *et al.*, 2004; Jiang *et al.*, 2010). The C57BL/6 strain is the most resistant to an infection, followed by BALB/c and C3H/HeN. Pro-inflammatory cytokines are secreted early on during an infection in the C57BL/6 strain, which coincides with the rapid eradication of a chlamydial infection. The more susceptible mouse strains C3H/HeN and BALB/c however, display a delayed and prolonged induction of pro-inflammatory cytokines, a longer course of infection and more severe pathology by comparison to C57BL/6 mice (Darville *et al.*, 2001; Jiang *et al.*, 2010). Furthermore, the involvement of PRRs also differs considerably following genital and respiratory infections (Darville *et al.*, 2003; Naiki *et al.*, 2005; Welter-Stahl *et al.*, 2006; Ouburg *et al.*, 2009; Zhang *et al.*, 2009; Chen *et al.*, 2010; Derbigny *et al.*, 2010; He *et al.*, 2011; Nagarajan *et al.*, 2011; O'Connell *et al.*, 2011; Derbigny *et al.*, 2012).

Despite their activation, Nod1, TLR4 and TLR9 have no effect on bacterial burden or pathology following a genital tract infection (Darville *et al.*, 2003; Welter-Stahl *et al.*, 2006; Ouburg *et al.*, 2009). *C. muridarum* was found to be a strong inducer of tissue-damaging IFNβ by oviduct epithelial cell lines in a toll/interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon-β (TRIF)- and interferon regulatory transcription factor (IRF) 3-dependent manner (Derbigny *et al.*, 2010; Derbigny *et al.*, 2012). Therefore, TLR3 may contribute to the development of pathology in mice following a genital tract infection, although this is yet to be shown *in vivo*. Mice deficient in TLR2 have a comparable course of infection to WT mice, but have significantly reduced upper reproductive tract tissue damage (Darville *et al.*, 2003). Moreover, the plasmid-cured strain of *C. muridarum* has a decreased ability to activated TLR2 (O'Connell *et al.*, 2011). Wild-type mice infected with plasmid-free *C. muridarum* display a similar course to TLR2-deficient mice infected with WT *C. muridarum* (O'Connell *et al.*, 2011), indicating a strong pathological role for TLR2 following a genital tract infection. The myeloid differentiation primary response gene (MyD) 88, downstream of all TLR's except TLR3, is essential for nuclear factor-κ-B

(NFκB) signalling and transcription of pro-inflammatory cytokines. MyD88$^{-/-}$ mice exhibit delayed clearance and develop severe pathology compared to WT animals following a genital tract infection, which was attributed to a decreased ability to prime the adaptive immune response (Chen *et al.*, 2010; Nagarajan *et al.*, 2011).

Contrastingly to the effects seen in the genital tract model, TLR2 inhibits severe lung inflammation by suppressing the same pro-inflammatory response over-expressed and deemed damaging during a genital tract infection (He *et al.*, 2011). Similarly with the genital tract model, MyD88-deficient mice have exaggerated respiratory tract infection compared to WT mice (Zhang *et al.*, 2009). These mice not only display a reduced level of cytokines and chemokines, but also an impaired ability to recruit and initiate the adaptive response. The innate response therefore mediates the development of pathology and generation of the adaptive immune response following a chlamydial infection, in a site-specific manner, by regulating host cytokine and chemokines secretions (Rasmussen *et al.*, 1997; Johnson, 2004; Watanabe *et al.*, 2004).

Epithelial cells

Infected murine cells secrete a myriad of cytokines and chemokines, including IL-1β, TNFα, IL-6, IL-8 and GM-CSF, in response to a *C. muridarum* infection (Darville *et al.*, 2001; Rank *et al.*, 2010) similar to that seen in humans (Agrawal *et al.*, 2009). Although epithelial cells have the capacity to mediate a degree of self-protection against infection, the epithelium primary acts as a surveillance system, recruiting immune cells better capable of eradicating the infection. However, pro-inflammatory cytokines/chemokines secreted by *Chlamydia*-infected epithelial cells are the hypothesised drivers of pathology in the cellular paradigm of pathogenesis (Hogquist *et al.*, 1991; Zhang and Phan, 1996; Perfettini *et al.*, 2000; Stephens, 2003; Hvid *et al.*, 2007; Darville and Hiltke, 2010).

Neutrophils

Neutrophils are recruited early in massive numbers in the mouse during the acute inflammation phase, similar to that seen in humans (Ripa *et al.*, 1979; Kiviat *et al.*, 1990; Molestina *et al.*, 1999; Geisler *et al.*, 2005; Ficarra *et al.*, 2008; Patel *et al.*,

2010). Characterised as professional phagocytic cells, neutrophils remove microbial products and other cellular debris, but also mediate tissue repair and remodelling. Neutrophils exert killing activity predominantly by production of superoxides, reactive nitrogen species, proteolytic enzymes and antimicrobial peptides (Bratton and Henson, 2011). Neutrophils can also discharge filaments of deoxyribonucleic acid (DNA) and chromatin, bound with antimicrobial compounds known as neutrophil extracellular traps (NET), to contain the spread of infection and activate T cells (Tillack *et al.*, 2012). There is no doubt that neutrophils control *Chlamydia* in the early stages of a genital tract infection, as neutrophil depletion increases chlamydial progenies during the preliminary rounds of replication (Barteneva *et al.*, 1996; Rank *et al.*, 2011). It has been suggested however that neutrophils may also facilitate the spread of an infection by actively "sluffing" and rupturing the infected epithelium (Rank *et al.*, 2008; Rank *et al.*, 2011). The longevity and degree of neutrophil influx has long been associated with the development of upper genital tract pathology (Darville *et al.*, 1997; Shah *et al.*, 2005b). Mice deficient in the chemokine receptor CXCR2, which mediates acute inflammation and neutrophil recruitment, develop significantly lower rates of infertility compared to WT animals (Lee *et al.*, 2010a). Animals infected with the plasmid-deficient strain of *C. muridarum* (CM3.1), secrete less chemotatic factors, recruit less neutrophils and show increased neutrophil apoptosis compared to animals infected with the WT strain (Frazer *et al.*, 2011). Despite displaying a normal course of infection, CM3.1-infected animals are relatively impervious to pathology, which further implicates the pathological role of neutrophils. Moreover, neutrophil degranulation products such as MMP9 and reactive oxygen species (ROS) have also been shown to contribute towards pathology (Ramsey *et al.*, 2001a; Ramsey *et al.*, 2005).

Neutrophils control an infection and enhance the development of pathology following a genital tract challenge. However, neutrophils appear to contribute more to disease than infection resolution in the respiratory tract model of infection. Mouse strains BALB/c and C3H/HeN mice are more susceptible to infection and pathology following a respiratory tract infection than the resistant C57BL/6 strain. BALB/c and C3H/HeN mice show a greater influx of neutrophils during a respiratory tract infection, suggesting neutrophils are not only inefficient at controlling the infection

but also causally linked with severity of pathology (Bai *et al.*, 2005; Tang *et al.*, 2009; Jiang *et al.*, 2010). *C. pneumoniae* has been shown to infect neutrophils and delay apoptosis (van Zandbergen *et al.*, 2004), which in the genital tract model has been associated with tissue damage (Barteneva *et al.*, 1996). Recruitment of neutrophils by *C. pneumoniae* has also been suggested to enhance replication in epithelial cells (Rodriguez *et al.*, 2005). Therefore, neutrophils play a complex yet predominantly pathological role during chlamydial infection in mice as they do in humans (Wiesenfeld *et al.*, 2002; Patel *et al.*, 2010).

Macrophages

Macrophages are also professional phagocytic cells, equipped with a similar antimicrobial arsenal to neutrophils, but with the additional ability to process and present antigens to T cells. Despite the ability to generate the adaptive immune response, macrophages are also significant contributors towards immunopathology in mice. Macrophages and neutrophils are the major sources of IL-1β, which elicits modest protection from a genital tract infection, but also has a strong correlation with tubal factor infertility in mice and humans (Hvid *et al.*, 2007; Agrawal *et al.*, 2009; Prantner *et al.*, 2009; Nagarajan *et al.*, 2012). Caspase-1 cleaves pro-IL-1β to the mature and biologically active form of IL-1β (Thornberry *et al.*, 1992). Capase-$1^{-/-}$ mice exhibit a normal primary and secondary course of infection, but significantly less pathology than WT animals (Cheng *et al.*, 2008). Activation of caspase-1 and secretion of active IL-1β by macrophages *in vitro* is stimulated through the NLRP3 inflammasome (Abdul-Sater *et al.*, 2010). However, depletion of NLRP3 and the crucial adaptor molecule apoptosis associated speck-like protein containing caspase recruitment domain (ASC), had no significant effect on IL-1β levels or the development of pathology *in vivo* (Nagarajan *et al.*, 2012), indicating the redundancy of the inflammasome in IL-1β-induced pathology. Macrophages also express nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and iNOS, responsible for the generation of antimicrobial oxygen and nitrogen free radicals, respectively. These free radicals have contrasting roles in the development of chronic chlamydial disease, despite lacking any effect on infection. Reactive nitrogen species (RNS) prevent chronic disease by regulating ROS, which activate MMPs responsible for extracellular matrix remodelling and induction of tissue scarring (Ramsey *et al.*,

2001b). However, RNS secretions by macrophages have also been shown to increase the risk of infertility by transiently disrupting the oviduct pacemaker, responsible for clearing oviduct secretions (Dixon *et al.*, 2010).

Fewer studies have looked at the role of macrophages during a lung infection. Alveolar macrophages have been shown to restrict the growth of *C. muridarum* (Qiu *et al.*, 2008). $IFNRA^{-/-}$ mice were also shown to be significantly more resistant to infection and develop less lung pathology, due to a greater influx and less apoptosis of macrophages (Qiu *et al.*, 2008). Interestingly, caspase-1 and IL-1β are critical for host defence against *C. pneumoniae* infection and pathology, contrasting with the effects in the genital tract infection model (Cheng *et al.*, 2008; Prantner *et al.*, 2009). Caspase-1-deficient mice had delayed cytokine production and reduced iNOS levels in alveolar macrophages. Macrophages have been suggested to contribute to persistence and the spread of systemic *C. pneumoniae* in mice and humans (Beagley *et al.*, 2009a). Therefore, macrophages appear to have contrasting protective/pathological roles in mice during chlamydial respiratory and reproductive tract infections, respectively. It is important to point out that this section only discussed "classically-activated" macrophages (M1), which secrete microbicides and are associated with DTH and tissue damage (Gordon, 2003). Currently there is no information regarding the anti-inflammatory "alternative-activated" macrophages (M2), which secrete arginases and facilitate tissue repair (Gordon, 2003), in the context of a chlamydial infection.

Natural killer cells cells

These cells have three main functions, cytotoxic lysis, cytokine and chemokine secretion, and contact-dependent co-stimulation (Peixoto deToledo *et al.*, 2009). Natural killer cells are recruited following a genital tract infection, in a MyD88-dependent manner (Nagarajan *et al.*, 2011), recognise/lyse *Chlamydia*-infected cells and secrete large amounts of IFNγ (Williams *et al.*, 1997; Tseng and Rank, 1998). This response efficiently reduces bacterial burden by polarising a pro-inflammatory Th1-type immune response (Tseng and Rank, 1998). The role NK cells play in pathogenesis however, has not yet been discussed in the context of the genital tract model.

Similarly, *C. muridarum* stimulates NK cells to produce IFNγ following a respiratory tract challenge (Williams *et al.*, 1993; Han *et al.*, 2008; Zhao *et al.*, 2011). Their involvement in the control of infection was initially thought to be minimal (Williams *et al.*, 1987), although a subsequent study has indicated that NK depletion exacerbates disease and infection, by significantly affecting DC maturation (Jiao *et al.*, 2011). Natural killer cells also prevent the *Chlamydia*-induced Th2-driven allergic-sensitisation response commonly associated with asthma (Han *et al.*, 2008). Therefore, NK cells play a predominantly protective role against chlamydial infection and disease.

The adaptive immune response

Adaptive immunity is characterised by an ability to develop and retain immunological memory. This is divided into the T cell-driven cell-mediated and the antibody-producing B cell responses. Generally, the cell-mediated responses targets intracellular pathogens and the humoral response drives antibody production, responsible for the elimination of extracellular pathogens (Guerra-Infante *et al.*, 1999; Mathews *et al.*, 2001; Debattista *et al.*, 2003). As an active chlamydial infection involves the presence of both the extracellular EBs and intracellular RBs, protection theoretically requires a balance between the antibody and cell-mediated responses. The importance of generating T and B cell responses against *Chlamydia* is clearly evident in severely immuno-compromised mice. Severe-combined immunodeficiency (SCID) and athymic nude mice, deficient in cell-mediated and antibody responses (except IgM), develop chronic *Chlamydia* infections (Rank *et al.*, 1985; Cotter *et al.*, 1997b; Hawkins *et al.*, 2002). Adoptive transfer of *Chlamydia*-specific T cells clear unresolved infections in mice with compromised cell-mediated responses (Ramsey and Rank, 1991; Thoma-Uszynski *et al.*, 1998) and enhance infection eradication in immune competent animals (Su and Caldwell, 1995; Morrison *et al.*, 2000; Morrison *et al.*, 2011). Passive immunisation with convalescent serum or monoclonal antibodies has also proven effective against preventing a re-infection and pathology (Pal *et al.*, 1997b; Su *et al.*, 1997; Murthy *et al.*, 2004; Pal *et al.*, 2008). Therefore, development of T cell- and B cell-driven adaptive immune responses following immunisation is crucial to eradicate an infection and prevent against re-infection, respectively (Farris and Morrison, 2011).

Despite some differences in the involvement of innate immune population in infection and pathology between infection models, there is little difference in the adaptive immune responses required to elicit protection against genital and respiratory infections.

T lymphocytes

T cells are defined by the expression of the T cell receptor (TCR) (αβ and γδ heterodimers) that recognises antigens presented on major histocompatability complexes (MHC). T cell receptor β-chain and not δ-chain-expressing T cells are required to clear an infection (Perry *et al.*, 1997), which includes the majority of helper, cytotoxic and regulatory T cell types. In addition to the antigen-specific signal from the TCR/MHC complex, effective T cell activation requires a second survival signal generated through surface co-receptors. Interaction between T cell co-stimulatory molecules (CD28, CD40L and inducible co-stimulatory molecule (ICOS)) and their reciprocal receptor/ligands (CD80/CD86, CD40 and inducible co-stimulatory ligand (ICOSL)) expressed on antigen-presenting cells (APCs), respectively, heavily influences protection against *Chlamydia* (Marks *et al.*, 2007; Chen *et al.*, 2009a; Kadkhoda *et al.*, 2010). Co-stimulatory molecules also function to polarise T cells into protective, non-protective or regulatory subsets (Smith *et al.*, 2003; Tang *et al.*, 2003; de Jong *et al.*, 2004).

$CD4^+$ T helper (Th) cells

MHC class II-restricted $CD4^+$ Th cells do not have a direct effector function, but instead "help" other immune cells to clear an infection by promoting antibody production, cytolytic and phagocytic activities. $CD4^+$ Th cells are the most important mediators of immunity against *Chlamydia* genital and respiratory infections and the associated pathology (Landers *et al.*, 1991; Ramsey and Rank, 1991; Su and Caldwell, 1995; Perry *et al.*, 1997; Thoma-Uszynski *et al.*, 1998; Morrison *et al.*, 2000; Smith *et al.*, 2003; Tang *et al.*, 2003; de Jong *et al.*, 2004; Marks *et al.*, 2007; Chen *et al.*, 2009a; Kadkhoda *et al.*, 2010; Morrison *et al.*, 2011; Peng *et al.*, 2011). Mice deficient in mature $CD4^+$ Th cells (MHC class $II^{-/-}$) (Grusby *et al.*, 1991) or with reduced Th activity ($CD4^{-/-}$) (Killeen *et al.*, 1993), either fail to resolve an infection or have delayed development of immunity (Morrison *et al.*, 1995).

Furthermore, the recruitment and maintenance of $CD4^+$ T cells at the site of infection coincides with infection resolution and dictates the longevity of immunity against re-infection, respectively (Igietseme and Rank, 1991; Igietseme *et al.*, 1999). The importance of $CD4^+$ T cells is also apparent in human studies (Kimani *et al.*, 1996; Van Voorhis *et al.*, 1997a; Debattista *et al.*, 2002; Kinnunen *et al.*, 2002; Geisler *et al.*, 2004; Agrawal *et al.*, 2007; Agrawal *et al.*, 2009).

Naive $CD4^+$ Th cells (Th0) diverge following clonal expansion into different Th subsets characterised by a unique signature of cytokines (O'Garra, 1998). Despite their plasticity, Th cell are conventionally assigned a type based on their production of IFNγ (Th1), IL-4 (Th2) or IL-17 (Th17) (Spellberg and Edwards, 2001; Harrington *et al.*, 2006). There are many different types of Th cells, but these three are important for chlamydial infections..

T helper type 1 cells

Identified by the production of IFNγ, $CD4^+$ Th1 cells are the dominant protective subset against genital and respiratory tract infections with *Chlamydia* (Igietseme *et al.*, 1993; Su and Caldwell, 1995; Cotter *et al.*, 1997b; Johansson *et al.*, 1997; Perry *et al.*, 1997; Lampe *et al.*, 1998; Igietseme *et al.*, 1999; Lu and Zhong, 1999; Wang *et al.*, 1999; Yang *et al.*, 1999; Yang, 2001; Rothfuchs *et al.*, 2004; Jayarapu *et al.*, 2009; Kadkhoda *et al.*, 2010). The generation of Th1 cells is dependent on IL-12 production by DCs. Adoptive transfer of *Chlamydia*-pulsed DCs secreting IL-12 prior to challenge elicit a Th1-biased response and induce strong immunity against a chlamydial genital tract infection and pathology (Su *et al.*, 1998). Similarly, intravenous transfer of IL-12p40$^{-/-}$ DCs fail to effectively prime a Th1-dominant response and are unable to elicit protection against a lung infection in mice when compared to WT DCs (Lu and Zhong, 1999). Lymphocytes isolated from mice deficient in IL-10, a negative regulator of the Th1 immunity, showed a significant induction of IL-12 and IFNγ following re-stimulation when compared to WT lymphocytes. IL-10-deficient mice displayed enhanced of protective immunity against infection and pathology in both challenge models (Yang *et al.*, 1999; Igietseme *et al.*, 2000). Therefore, production of IL-12 by DCs is crucial for

eradicating the bulk of bacterial burden through the recruitment, activation and polarisation of IFNγ-secreting $CD4^+$ Th1 cells (Perry *et al.*, 1997).

Interferon-γ is required to completely resolve and prevent systemic spread of a chronic chlamydial infection and the associated pathology (Rank *et al.*, 1992; Cotter *et al.*, 1997b; Perry *et al.*, 1997; Williams *et al.*, 1997; Wang *et al.*, 1999; Nelson *et al.*, 2005b; Jupelli *et al.*, 2008). Similarly, IFNγR-deficient mice exhibit greater chlamydial burden and dissemination of infection (Johansson *et al.*, 1997; Ito and Lyons, 1999; Jupelli *et al.*, 2010). Interferon-γ can stimulate numerous host cell defence mechanism including IDO, iNOS and iron deprivation, although activation of p47 GTPases are in part responsible for mediating protection in mice (Nelson *et al.*, 2005b; Coers *et al.*, 2011). Interferon-γ is also known to have a synergistic relationship with a number of different cytokines, including TNFα and IL-17 (Amber *et al.*, 1988; Shemer-Avni *et al.*, 1988; Holtmann *et al.*, 1990; Fichorova and Anderson, 1999; Robinson *et al.*, 2003; Gabr *et al.*, 2011). $CD4^+$ Th1 cells secreting multiple cytokines (eg. IFNγ and TNFα), have proven far more effective than single cytokine-producing T cells in conferring protection from various mucosal pathogens (Darrah *et al.*, 2007; Forbes *et al.*, 2008), including *Chlamydia* (Igietseme *et al.*, 1993; Olsen *et al.*, 2010; Yu *et al.*, 2010; Yu *et al.*, 2011; Yu *et al.*, 2012). Although the mechanism by which IFNγ elicits protection in mice is still unclear (Cotter *et al.*, 1997b; Coers *et al.*, 2011), the primary aim of a chlamydial vaccine is to elicit a robust Th1 response.

T helper type 2 cells

Interleukin-4-dependent $CD4^+$ Th2 cells are largely considered to be non-protective against *Chlamydia* (Wang *et al.*, 1999; Yang, 2001). T helper type 2 cytokines (IL-4, IL-5, IL-10, IL-13) have no influence on the clearance of an infection (Perry *et al.*, 1997; Williams *et al.*, 1997; Hawkins *et al.*, 2002) and are often associated with the exacerbation of pathology following a primary infection, due their suppressive effect on the Th1 response (Yang *et al.*, 1996; Perry *et al.*, 1997; Wang *et al.*, 1999; Kaiko *et al.*, 2008; Chen *et al.*, 2010; Asquith *et al.*, 2011). However, $CD4^+$ Th2 polarised cells aid in the production of serum and mucosal IgA antibodies (Morrison *et al.*, 1995; Su and Caldwell, 1995; Morrison and Morrison, 2000; Morrison *et al.*, 2000;

Hawkins *et al.*, 2002), necessary for protection against re-infection (Pal *et al.*, 1997b; Pal *et al.*, 2008). As negative regulators of the Th1 response, Th2 may also be involved in regulation (Debattista *et al.*, 2003), minimising the potential inflammatory damage inherent of Th1 over-expression (Buendia *et al.*, 2002). Therefore, a vaccine will also need to induce a Th2 response, at least to promote the production of antibodies.

T helper type 17 cells

Activated by IL-1β, IL-6 and IL-23, IL-17-secreting $CD4^+$ Th17 cells primarily recruit and activate neutrophils (Basso *et al.*, 2009; Korn *et al.*, 2009). The early MyD88-dependent induction of IL-17 is critical for protection against a respiratory tract challenge (Bai *et al.*, 2009; Zhang *et al.*, 2009), but this is most likely unrelated to the recruitment of neutrophils (Bai *et al.*, 2005). Mice treated with an IL-17-neutralising antibody display significantly greater weight loss, bacterial burden and pathological damage following respiratory tract infection compared to the isotype control treated animals (Bai *et al.*, 2009; Zhang *et al.*, 2009). Interleukin-17 does not have a direct anti-chlamydial effect *in vitro*, but enhances the production of other pro-inflammatory cytokines and chemokines responsible for recruiting other immune cells (Zhang *et al.*, 2009). Interleukin-17 also modulates the induction of IL-12 by DCs, which is thought to enhance protection by promoting Th1 immunity (Bai *et al.*, 2009; Scurlock *et al.*, 2010). However, enhanced expression of IL-17/IL-17R in the lung tissues has been linked with susceptibility to infection and disease in the respiratory tract model (Zhou *et al.*, 2009). Attempts to elucidate the role of IL-17 signalling in the genital tract model by KO of IL-17RA, resulted in the induction of a number of complex compensatory mechanisms (Scurlock *et al.*, 2010), suggesting that signalling through IL-17RA is redundant for normal resolution of infection. As IL-17 appears to exhibit both protective and pathological properties (Yu *et al.*, 2010; Lu *et al.*, 2011) the Th17 response may need to be avoided for a chlamydial vaccine.

T regulatory cells

Secreting IL-10 and transforming growth factor (TGF)-β, $CD4^+CD25^+FoxP3^+$ T_{reg} cells are most well known for their induction of tolerance. Inducible T_{reg} also control collateral tissue damage caused by a vigorous pro-inflammatory response in a

contact-dependent and -independent manner (Curotto de Lafaille and Lafaille, 2009; Jiang and Kelly, 2011). Currently, the role T_{reg} play during a *Chlamydia* infection is unknown. However, expression of the T_{reg} transcription factor forkhead box P3 (FoxP3) mirrors the increase in expression of T-bet (Th1 transcription factor) in the genital tract following an infection (Moniz *et al.*, 2010; Marks *et al.*, 2011b). This may indicate that T_{reg} are responsible for controlling Th1 immunity and suppression of tissue damage in the genital tract model. Mice deficient in ICOS, present on T cells, have also been implicated T_{reg} in the control of Th1-driven immunopathology following a genital tract challenge (Marks *et al.*, 2007). In the absence of ICOS, mice displayed enhanced Th1 immunity and infection resolution, but also exacerbated pathology. This coincided with an impaired development of T_{reg} and IL-10 expression. Although T_{reg} also appear to play a similar role in the respiratory tract model, controlling Th1 responses and preventing the onset of the allergic sensitisation common in asthma (Crother *et al.*, 2011; Wantia *et al.*, 2011), it is unclear what role T_{reg} could play in a chlamydial vaccine-induced response.

$CD8^+$ cytotoxic lymphocytes (CTLs)

The primary function of $CD8^+$ T cells is to mediate the destruction of infected cells. *Chlamydia*-specific $CD8^+$ T cells can recognise and lyse infected epithelial cells *in vitro* (Lampe *et al.*, 1998), although CTL-mediated perforin-induced cytolysis has no significant impact on the resolution of a genital tract infection and may in fact exacerbate oviduct pathology (Morrison *et al.*, 1995; Perry *et al.*, 1999a; Morrison *et al.*, 2000; Morrison and Morrison, 2001; Murthy *et al.*, 2011a). Similarly, CTLs also play a subordinate role against *C. muridarum* in the respiratory tract model (Magee *et al.*, 1995; Williams *et al.*, 1997). The poor protection induced by CTLs may be in part due to an inhibition of chlamydial antigen presentation by the infected epithelium, due to the elusive intracellular chlamydial vesicles and the immunosuppressive affect of the chlamydial protease-like activity factor (CPAF) on MHC molecule expression (Su and Caldwell, 1995; Zhong *et al.*, 2000). Some studies have reported protection against a chlamydial infection following the transfer of $CD8^+$ T cells, although the heightened immunity was suggested to be driven by the production of pro-inflammatory cytokines (IFNγ and TNFα) and not the cytolytic activity (Igietseme *et al.*, 1994; Starnbach *et al.*, 1994; Thoma-Uszynski *et al.*, 1998;

Rothfuchs *et al.*, 2004). Therefore, MHC class I-restricted $CD8^+$ CTLs exhibit both protective and pathological roles against *Chlamydia*, determined largely by specific cytokine secretions (Igietseme *et al.*, 1994; Murthy *et al.*, 2011a).

B lymphocytes

B cells can act as APCs, but protect primarily through the production of antibodies. B cell-deficient mice ($\mu MT^{-/-}$ and $Igh^{-/-}$) resolve a primary genital tract infection similar to WT animals, but are significantly more susceptible to rechallenge (Su *et al.*, 1997; Williams *et al.*, 1997). B cells are also crucial to prevent both a primary and secondary respiratory tract infection, particularly through the induction of T cell responses (Yang and Brunham, 1998). Immunoglobulin A and IgG neutralise *Chlamydia in vitro* (Pal *et al.*, 1997b; Peterson *et al.*, 1998) and increased *Chlamydia*-specific antibody titres associate directly with improved clearance and reduced pathology *in vivo* (Morrison *et al.*, 1995; Su *et al.*, 1997; Yang and Brunham, 1998; Murthy *et al.*, 2004; Morrison and Morrison, 2005; Rodriguez *et al.*, 2006). Immunoglobulin A is thought to reduce infection by directly preventing chlamydial attachment to the host cells, as KO of the polymeric immunoglobulin receptor (pIgR), responsible for receptor-mediated transport of IgA into the lumen, significantly increases chlamydial infectivity *in vivo* (Cunningham *et al.*, 2008). The *Chlamydia*-neutralising potential of IgG was found to be independent of aggregation, the induction of complement or inhibition of attachment *in vitro* (Caldwell and Perry, 1982). Mice deficient in antibody-binding Fc receptors show an increase in bacterial burden after genital tract re-infection, indicating the potential involvement of IgG-dependent cellular cytotoxicity (ADCC), opsonisation and engulfment by macrophages (Moore *et al.*, 2002). Anti-chlamydial antibodies also enhance antigen presentation for the induction of a Th1 response and early clearance of infection in an Fc-dependent manner (Moore *et al.*, 2002). Interestingly, it has also been suggested that IgG may neutralise an infection intracellularly following the internalisation of *Chlamydia* and the assembly of the inclusion membrane (Caldwell and Perry, 1982).

In summary, key aspects of the cell-mediated response, namely the MHC class II-restricted $CD4^+$ Th1 (Morrison *et al.*, 1995; Penttila *et al.*, 1998; Yang *et al.*, 1998)

and the pro-inflammatory cytokines IFNγ (Cotter *et al.*, 1997b; Lampe *et al.*, 1998; Rothfuchs *et al.*, 2004) and IL-12 (Perry *et al.*, 1997; Lu and Zhong, 1999) are the major defensive mechanisms against primary and secondary *Chlamydia* infections and the development of pathology in both mice and humans (Kimani *et al.*, 1996; Van Voorhis *et al.*, 1997a; Debattista *et al.*, 2002; Kinnunen *et al.*, 2002; Geisler *et al.*, 2004; Agrawal *et al.*, 2007; Agrawal *et al.*, 2009). Antibodies, particularly secretory IgA, have also been directly linked with the reduction of chlamydial shedding, resistance against re-infection and prevention of pathology (Su *et al.*, 1997; Yang and Brunham, 1998; Morrison and Morrison, 2005). The contribution of each immune cell type to protective immunity is still widely disputed (Rank and Batteiger, 1989; Penttila *et al.*, 1998; Johansson and Lycke, 2001; Morrison and Morrison, 2001; Morrison and Morrison, 2005; Nelson *et al.*, 2005b). The overwhelming consensus is however that an efficacious vaccine, protective against both genital and respiratory tract challenges must generate a strong Th1 cell-mediated response with an accessory mucosal antibody response, capable of preventing the establishment and transmission of infection as well as pathology (Beagley and Timms, 2000; Longbottom and Livingstone, 2006).

HISTORY OF CHLAMYDIAL VACCINE DEVELOPMENT

Whole organism vaccines

First generation vaccines, consisting of live attenuated or inactivated whole organisms, form the basis of some of the most effective human vaccines (eg. smallpox and yellow fever). The protection against infection conferred following immunisation with growth attenuated *Chlamydia* strains in animal models has shown the potential benefit of a live attenuated vaccine for the human population (Rodolakis and Bernard, 1984; Su *et al.*, 2000). Unfortunately, no growth attenuated strains of *C. pneumoniae* or *C. trachomatis* exist due to an inability to genetically manipulate *Chlamydia*, which until recently did not exist (Wang *et al.*, 2011b). Most studies have instead utilised virulence attenuated (avirulent) strains of *Chlamydia* to assess the protective capabilities of an attenuated vaccine. Avirulent chlamydial strains that are cured of the "cryptic" plasmid have similar growth characteristics as the WT strain, but cause minimal disease following an infection (O'Connell *et al.*, 2007).

Mice immunised with the *C. muridarum* plasmid-cured strain CM3.1, were partially protected against repeated challenges with the homologous virulent strain, but did not develop adverse upper reproductive tract pathology (O'Connell *et al.*, 2007). Similar results were obtained with the plasmid-free human biovar *C. trachomatis* L2 (25667R) against a genital infection in the mouse (Olivares-Zavaleta *et al.*, 2010) and the plasmid-deficient *C. trachomatis* serovar A (A2497P^{-}) against an ocular infection in non-human primates (Kari *et al.*, 2011). However, this approach for a human vaccine has a number of short-comings. Attenuated vaccines using avirluent strains elicit a serovar-specific response and provide no immunity against subsequent challenges with non-homologous strains (Olivares-Zavaleta *et al.*, 2010). Curing *Chlamydia* of the cryptic plasmid also does not always induce an avirluent phenotype in all chlamydial species or animal models (Frazer *et al.*, 2012). Furthermore, immunisation with an attenuated vaccine could prime recipients for pathology, similar to how patients with a history of repeated infections are more likely to develop pathology (immunological paradigm) (Kimani *et al.*, 1996; Hillis *et al.*, 1997; Brunham and Rey-Ladino, 2005; Bakken *et al.*, 2007), potentially by generating a self-reactive adaptive response (eg. HSP60-specific).

Whole inactivated organism vaccines against *C. felis* and *C. abortus*, which reduce acute disease (abortion), are commercially available for veterinary use (Longbottom and Livingstone, 2006). The protection elicited by these whole organism vaccines however, is short-lived, serovar-specific and unable to prevent colonisation, chlamydial shedding or the secondary sequelae associated with chronic infection (Longbottom and Livingstone, 2006). Moreover, immunisation with whole inactivated organism vaccines has been shown in some instances to exacerbate pathology. Early whole inactivated vaccines against trachoma, the ocular chlamydial infection causing blindness, enhanced DTH reaction and the severity of disease in some patients (Grayston *et al.*, 1963; Dhir *et al.*, 1967; Woolridge *et al.*, 1967). These findings were later replicated in non-human primate models (Collier *et al.*, 1967; Wang *et al.*, 1967; MacDonald *et al.*, 1984), which suggested that *Chlamydia* contain both immunoprotective and immunopathological antigens. Consequently, a whole organism approach towards a human vaccine has largely been abandoned and

the predominant focus of vaccine development today has shifted towards second generation subunit vaccines.

Subunit vaccines

Subunit vaccines generally consist of microbial components, such as proteins, peptides, polysaccharides or DNA, as opposed to the whole intact organism. Protein-based vaccines have consistently induced greater protection against chlamydial infections than any other microbial subunits (Brunham and Rey-Ladino, 2005; Farris and Morrison, 2011). Subunit vaccines have been trialled using chlamydial outer membrane proteins, type III secretion system (T3SS) effector proteins, secretory and inclusion membrane proteins, given individually or in combination with varying success (Pal *et al.*, 2005; Li *et al.*, 2007; Sun *et al.*, 2009; Wang *et al.*, 2009). The chlamydial MOMP is currently one of the strongest subunit vaccine candidates. The MOMP constitutes approximately 60% of the chlamydial outer membrane protein mass (Brunham and Peeling, 1994) and contributes largely to the structural integrity of *Chlamydia*. Unlike T3SS (Tarp), secretory (CPAF) and inclusion membrane proteins (Inc's), the MOMP is expressed throughout all stages of the developmental cycle (Belland *et al.*, 2003). Therefore, MOMP-specific immunity can recognise EBs, RBs and even the persistent abberant form (Hogan *et al.*, 2004), and target *Chlamydia* for immune destruction at all stages of replication (Igietseme *et al.*, 2005). The MOMP also elicits protective cell-mediated and antibody responses (Farris *et al.*, 2010), identical to that required to prevent and eradicate a *Chlamydia* infection. Immunisation with the MOMP has proven protective against infection and pathology in a number of animal models, including the mouse, guinea pig, pig, koala and non-human primate (Berry *et al.*, 2004; Skelding *et al.*, 2006; Sun *et al.*, 2009; Andrew *et al.*, 2011; Cheng *et al.*, 2011b; Schautteet *et al.*, 2011; Kollipara *et al.*, 2012; Schautteet *et al.*, 2012). A disadvantage of MOMP-based vaccination is that it often generates serovar-specific immunity that is primarily directed towards four surface exposed highly variable domains (Ramsey *et al.*, 2009). Some cross protection may exist within chlamydial serogroups (Zhang *et al.*, 1987; Zhang *et al.*, 1989), although a MOMP-based chlamydial vaccine may require a polyvalent approach encompassing antigens isolated from the most prevalent serovars in the target human population (Mossman *et al.*, 2008). Alternatively, a multi-subunit

vaccine may be adopted, which contains the MOMP together with antigens highly conserved amongst *Chlamydiales*. The greatest limitation of any subunit vaccine however is often their inability to generate long-lasting immunity following immunisation. Unlike whole organism vaccines that can be self-replicating and -adjuvanting, subunit vaccines will require better adjuvants and delivery systems to boost immunity, if they're to elicit greater protection than a natural infection (Igietseme *et al.*, 2005). Protection against infection is also reliant on the induction of a mucosal response. Therefore, a vaccine must also be delivered by an appropriate route of immunisation, capable of eliciting a localised immune response at the anatomical portal of entry of the invading pathogen.

ROUTES OF IMMUNISATION

Vaccines are traditionally administered via systemic routes (intramuscular (IM) or subcutaneous (SC)) using needles. The majority of parenteral vaccines currently approved for use in humans prevent infections caused by invasive mucosal pathogens (e.g. human papillomavirus (HPV) or influenza) through the induction of neutralising serum antibodies (Smith *et al.*, 2012). However, vaccines that elicit systemic humoral immunity alone are unable to protect against *Chlamydia* (Su *et al.*, 1995; Cheng *et al.*, 2011a). Systemic vaccines that induce a greater cell-mediated response improve protection against *Chlamydia* infection, although immunity still remained partial (Pal *et al.*, 1997a; Pal *et al.*, 2001). Alternatively, studies that initially primed animals via a mucosal route then boosted them systemically have shown far greater protection against *Chlamydia* than the parenteral vaccine alone (Ralli-Jain *et al.*, 2010). This is thought to be because parenteral vaccines are relatively poor inducers of localised mucosal responses (Muszkat *et al.*, 2000; Holmgren and Czerkinsky, 2005; Cong *et al.*, 2007; Pedersen *et al.*, 2011) that are vital for protection against pathogens like *Chlamydia*, which enter through and are predominantly confined to the epithelium.

The underlying mechanisms of how mucosal immunity develops is still unclear, although it appears to be determined by the micro-environment of the immunisation site (Chang and Kweon, 2010), the phenotype of the resident APCs (Dudda *et al.*, 2005) and the tissue-selective polarisation of the draining lymph nodes (Campbell

and Butcher, 2002; Calzascia *et al.*, 2005). For example, induction of T cell homing to the gastrointestinal tract is dependent on the secretion of retinoic acid by DCs during antigen presentation (Strober, 2008). Dendritic cells that secrete retinoic acid develop this phenotype in the gut by metabolising Vitamin A, which is absorbed in the gastrointestinal tract from food (Strober, 2008). A similar mechanism influences T cell homing to the skin, although this is mediated instead by DCs that have metabolised sunlight-derived Vitamin D (Sigmundsdottir *et al.*, 2007). Lymph nodes draining the epithelium also imprint their own unique pattern of selectins (P- and L-selectins) and integrins (α_4, β_1, β_7, $\alpha_4\beta_7$, and $\alpha_E\beta_7$) on activated lymphocytes during clonal expansion (Calzascia *et al.*, 2005), which often favours homing to mucosal surfaces. Systemic immunisation on the other hand down-regulates mucosa-associated homing receptors (Campbell and Butcher, 2002), essential for recruitment to mucosal surfaces at times of infection. Therefore, vaccines targeted to the epithelium elicit mucosal immunity by stimulating innate cell populations preconditioned to generate immune responses at mucosal surfaces (Brokstad *et al.*, 2002; Holmgren and Czerkinsky, 2005).

Every epithelium displays a unique pattern of selectins and addressins that acts as an area code for passing lymphocytes. The homing profiles imprinted on lymphocytes by tissue-resident APCs following mucosal immunisation, direct activated cells back to the epithelium where the initial antigen interaction occurred by recognition of this code. Many mucosal tissues however express similar patterns of adhesion molecules and chemokines. Therefore, lymphocytes activated following mucosal immunisation are often distributed amongst a variety of mucosal effector site throughout the body, in addition to the location where the antigen was encountered initially (Smith *et al.*, 2012). This is often referred to as the common-mucosal immune system (CMIS) (Figure 2.5). For example, a previous respiratory tract infection with *Chlamydia* protects mice against a subsequent lung infection, but also a genital tract challenge (Pal *et al.*, 1996). This distribution of protection relies on a distinct homing pattern imprinted on T and B cells (lymphocyte function-associated antigen (LFA)-1 and $\alpha_4\beta_7$) and expression of their reciprocal mucosal addressins (ICAM-1, MAdCAM-1 and VCAM-1) by epithelial and endothelial cells at both sites of infection (Kelly and Rank, 1997; Perry *et al.*, 1998; Kelly *et al.*, 2000; Kelly *et al.*, 2001; Kelly *et al.*,

2009). As the homing potential of circulating lymphocytes is dependent on the site of activation (Quiding-Jarbrink *et al.*, 1995; Kantele *et al.*, 1997; Quiding-Jarbrink *et al.*, 1997), choosing a route of immunisation that targets the epithelium and favours immunity at the major sites of chlamydial infection would improve the utility of a vaccine against *Chlamydia* immensely. We have selected three routes of non-invasive vaccine delivery that not only direct immunity to the desired mucosal compartments in a variety of ways (Quiding-Jarbrink *et al.*, 1997; Sigmundsdottir and Butcher, 2008), but are also approved for human treatment in some form or another (Mathias and Hussain, 2009).

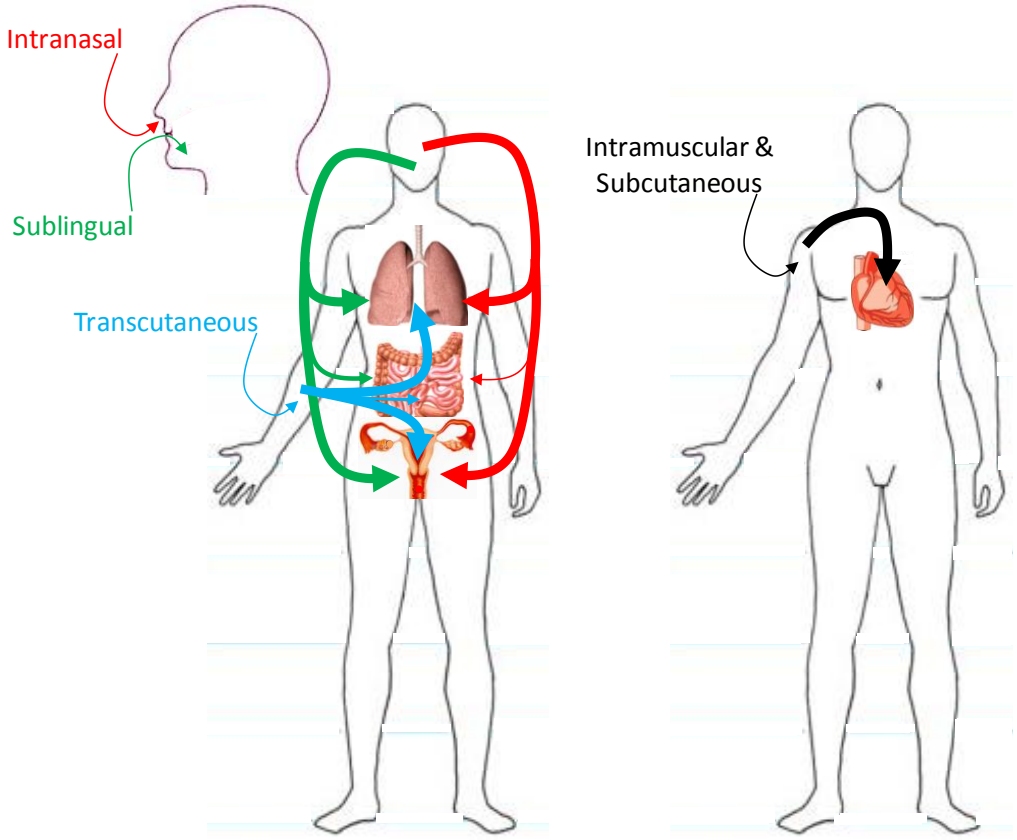


Figure 2.5: Illustration of the CMIS.

Mucosal [IN (red) and SL (green)] and cutaneous [TC (blue)] immunisation promote homing of activated lymphocytes to the inductive site and distant mucosal effector sites, like the respiratory, gastrointestinal and reproductive tracts. The thicker the arrow, the greater the potential of lymphocytes activated following immunisation to home to the indicated tissue. Parenteral immunisation via the IM and SC routes promotes systemic immunity, largely confined within the circulatory systems. Systemic immunisation can elicit mucosal IgG, although the source of this is from serum rather than the result of local production (Czerkinsky and Holmgren, 2010a; Czerkinsky and Holmgren, 2010b).

Transcutaneous immunisation

Transcutaneous immunisation involves the application of a vaccine directly onto intact skin. This route targets professional APCs called Langerhans' cells (LC) and dermal DCs that densely pack the epidermis and dermis at levels greater than 100,000 cells/cm^2 (Chen *et al.,* 2009b), making the skin a highly attractive route for vaccine delivery. The programming and homing of protective immunity following TC immunisation was shown to be intrinsically linked to sunlight-induced Vitamin D3 by resident DCs in the skin (Sigmundsdottir *et al.,* 2007). Skin-derived DCs that have metabolised Vitamin D3 promote expression of the chemokine receptor CCR10 on naive T cells upon antigen presentation, which can recruit cells via a CCL27/CCL28 chemokine gradient established by the genital and lung epithelia at times of inflammation (Lazarus *et al.,* 2003; Cha *et al.,* 2011). There have been a number of human clinical trials of TC vaccines that have reported protection against enteric and respiratory pathogens (Li *et al.,* 2011a). Some studies even found superior cell-mediated and mucosal antibody responses following TC vaccination than those elicited by more traditional administration routes like IM or SC (Etchart *et al.,* 2007; Combadiere *et al.,* 2010). Most importantly, no severe adverse reactions were noted in the recipients following any of the vaccine trials (Guerena-Burgueno *et al.,* 2002; Glenn *et al.,* 2003; Etchart *et al.,* 2007; Frech *et al.,* 2008; Hirobe *et al.,* 2012), which indicates that TC immunisation is protective as well as safe in humans. Transcutaneous immunisation has also been found to elicit mucosal responses and induce protection against genital and respiratory tract infections in animal models. In the context of a chlamydial vaccine, TC immunisation with the MOMP improved infection resolution in both the respiratory and reproductive challenge models (Gockel *et al.,* 2000; Berry *et al.,* 2004; Skelding *et al.,* 2006). Animals displayed enhanced IFNγ-expressing cell-mediated responses in the lymph nodes draining both sites of infection and the presence of antibody-secreting cells in the lung and vaginal tissues (Berry *et al.,* 2004; Skelding *et al.,* 2006; Hickey *et al.,* 2009). This indicates that the TC route is highly effective at promoting mucosal immunity in animal and human models. The greatest impediment to vaccines delivered via the TC route however is the poor permeability of the stratum corneum, which restricts the uptake of macromolecules greater than 500Da in size (Bos and Meinardi, 2000). Both

physical and chemical methods have however been used successfully to remove and/or penetrate the upper stratified layers (Warger *et al.*, 2007). Immune responses induced following TC delivery can be improved further through the recruitment of even greater numbers of APCs with chemoattractants (Hickey *et al.*, 2005) and by increasing the exposure time to vaccine constituents (Naito *et al.*, 2007).

Sublingual immunisation

More commonly used for delivery of low-molecular weight drugs and allergen-specific immunotherapy (Zhang *et al.*, 2002; Leatherman *et al.*, 2007), SL delivery, applied underneath the tongue, is a relatively new route yet to be trialled extensively in humans for a vaccine delivery. Immune cells of lingual tissue, recently mapped in detail in mice (Mascarell *et al.*, 2009), closely resemble the type and distribution of APCs found in the skin (Kweon, 2011). However, the buccal mucosa is up to four thousand times more permeable than the skin (Sohi *et al.*, 2009), which makes for easier passage of vaccines directly into the surrounding tissues and hence a more attractive avenue for vaccine delivery. The dissemination of protective immunity initiated in the SL mucosa is dependent on $CCR7^+$ DCs, which prime T and B cells to migrate towards the chemokine CCL28, expressed by epithelial cells at numerous sites in the body including the reproductive and respiratory tracts (Song *et al.*, 2009; Czerkinsky and Holmgren, 2010a). A clinical trial of SL immunisation using the polyvalent vaccine Bactek®, significantly reduced recurrent respiratory tract infections in patients with altered immune responses by increasing levels of antigen-specific T cells (Alecsandru *et al.*, 2011). This study and others have also reported that SL immunisation in human is well tolerated and free of any adverse reactions (Grosclaude *et al.*, 2002; Agostinis *et al.*, 2005; Olaguibel and Alvarez Puebla, 2005; Agostinis *et al.*, 2008; Alecsandru *et al.*, 2011). Sublingual immunisation has also had great success in a variety of animal models (Amuguni *et al.*, 2011). Sublingual immunisation has been shown to elicit equivalent or superior immunity against HPV and influenza than systemic methods of vaccination in mice, due to a greater induction of local responses at the site of infection (Cuburu *et al.*, 2009; Shim *et al.*, 2011). Similar to TC immunisation, SL immunisation elicits broad cell-mediated ($CD4^+$ and $CD8^+$) and mucosal antibody (IgG and IgA) responses in the reproductive and respiratory tract tissues of mice (Saeki *et al.*, 2000; Cuburu *et al.*, 2007; Song *et*

al., 2008; Cuburu *et al.*, 2009; Song *et al.*, 2009; Domm *et al.*, 2011). The SL route has not yet been studied comprehensively in regards to a *Chlamydia* infection, despite its potential to improve mucosal immunity (Carmichael *et al.*, 2011).

Intranasal immunisation

Intranasal immunisation circumvents many of the limitations encountered by TC and SL delivery in targeting the nasopharynx-associated lymphoid tissue (NALT) (Waldeyer's ring in humans), which consists of highly vascularised and permeable epithelial localised micro-fold (M) cells (Bockman and Cooper, 1973; Kiyono and Fukuyama, 2004). Specialised in processing and presenting antigenic macromolecules to T and B cells for the induction of antigen-specific mucosal responses, M cells are excellent targets for improving uptake and efficiency of mucosal vaccines. IN immunisation induces expression of high levels of CCR10, $\alpha_4\beta_1$ and $\alpha_4\beta_7$ on activated lymphocytes, which can be recruited to the genital and respiratory tract by corresponding ligand/receptor (Kiyono and Fukuyama, 2004). Intranasal immunisation has been successful in protecting mice against *Chlamydia* genital and respiratory tract challenges. Protection was conferred following vaccination by the generation of IFNγ-secreting T lymphocytes, IgA/IgG2a class switched B lymphocytes and recruitment of antibody-secreting cells to the site of infection (Skelding *et al.*, 2006; Tammiruusu *et al.*, 2007; Sun *et al.*, 2009; Andrew *et al.*, 2011). Intransal vaccines represent a large proportion of the needle-free vaccines currently approved or in clinical trials in humans (Harper *et al.*, 2003; Bukreyev *et al.*, 2004), second only to orally administered vaccines (Slutter *et al.*, 2008). FluMist® is a trivalent live-attenuated influenza vaccine delivered intranasally, which provides a superior local IgA response in the nasal cavity (Boyce *et al.*, 1999) than the more commonly used inactivated influenza vaccine delivered parenterally (Brokstad *et al.*, 2002). Despite inducing significantly less influenza-specific serum antibodies than the traditional inactivated IM vaccine, both were comparable at preventing culture-positive influenza (Edwards *et al.*, 1994; Beyer *et al.*, 2002). Intranasal immunisation with a recombinant HPV16 L1 virus-like particle (VLP) vaccine administered IN was also found to induce secretory IgA antibodies in vaginal secretion of human female volunteers, whereas the IM route did not (Nardelli-Haefliger *et al.*, 2005). Similarly, vaginal IgG and IgA were also detected

following IN immunisation with HIV-1 gp160 (Pialoux *et al.*, 2008). Intranasal vaccination has been proven effective at inducing protective immune responses at various mucosal surfaces and is largely well tolerated in human trials, with the occasional mild case of rhinorrhea (Nichol *et al.*, 1999; Pialoux *et al.*, 2008). However, there have been some reports of facial paresis following IN immunisation, due to toxin-based mucosal adjuvants (discussed below) that have restricted the development of IN vaccines (Fujihashi *et al.*, 2002; Couch, 2004; Kiyono and Fukuyama, 2004; Mutsch *et al.*, 2004).

Mucosal and cutaneous immunisation offers numerous advantages over parenteral immunisation. Vaccines utilising needle-free routes are much safer than parenteral vaccines and often result in fewer side effects (Bennett *et al.*, 2002; Glenn *et al.*, 2003). Moreover, it has been reported that up to 50% of immunisation injections in developing countries are unsafe and between 20 – 80% of new cases of hepatitis B virus (HBV) can be attributed to incorrect injection practices (Simonsen *et al.*, 1999). Needle-free vaccines are also significantly more cost-effective than parenteral vaccines, if the added financial burden of training and employment of trained personnel and transmission of blood-borne disease is taken into account. The oral polio vaccine (OPV) alone has a lower cost ($6.33(USD)) than the injectable inactivated polio vaccine (IPV) ($8.42(USD)) (Miller *et al.*, 1996). Even if cases of vaccine-associated paralytic poliomyelitis (VAPP) caused by OPV (occurring in 1 in 750,000 immunisations (Racaniello, 2006)) are considered, the needle-free OPV is still the most cost-effective option (Miller *et al.*, 1996). Furthermore, the direct and indirect costs of parenteral vaccination in the developing world can increase the cost per injection by over 40-fold (Ekwueme *et al.*, 2002). Maximising community compliance and speed of immunisation is also vital to facilitate rapid immunisation of large populations (potentially during outbreaks) and enhance vaccine uptake critical for herd immunity (Giudice and Campbell, 2006). Most importantly, vaccines targeting the epithelium elicit mucosal immunity critical for protection against mucosal pathogens like *Chlamydia*. Despite their potential benefits only a handful of needle-free vaccines are licensed for human use, the majority of which are self-replicating or -adjuvanting whole organism vaccines (Slutter *et al.*, 2008; Smith *et al.*, 2012). A subunit approach, like that required for a chlamydial vaccine, needs a

powerful adjuvant to initiate and sustain immunological memory (Czerkinsky *et al.*, 1999; Fujihashi and McGhee, 2004). Similarly, vaccines targeted to the epithelium also require a potent adjuvant to overcome the induction of tolerance that occurs following mucosal exposure to an antigen.

MUCOSAL TOLERANCE

Mucosal tolerance is a state of reduced antigen-specific immunological responsiveness elicited by mucosal exposure to a given antigen. Evolved to suppress unwanted immune responses to foreign but innocuous antigens like food, dust and commensal micro-organisms, mucosal tolerance is characterised by deletion or clonal anergy of antigen-specific T cells and the generation of T_{reg} (Smith *et al.*, 2012). One key determinant identified for the induction of oral tolerance was the dosage of antigen fed. High antigen dosages were found to promote anergy/deletion of antigen-specific cells, whereas low doses preferentially induced TGFβ-secreting and immunosuppressive T_{reg} (Weiner *et al.*, 2011). Mucosal tolerance is also thought to be in part the consequence of antigen presentation in the absence of effective co-stimulation (Weiner *et al.*, 2011). Therefore, vaccines targeting the mucosal epithelium must promote the expression of co-stimulatory molecules to overcome immune suppression and effectively prime T cell for the induction of mucosal immunity.

ADJUVANTS

The term adjuvant is derived from the Latin word *adjuvare* meaning "to help" (Lycke, 2010). Adjuvants provide help by creating local non-specific inflammation or acting as depot, slowly releasing antigen. Both mechanisms recruit and mature APCs, critical to enhance their capacity to co-stimulate naive T cells and generate long-lived T cell immunity (Lycke, 2007). The importance of APCs maturation for optimal protection against *Chlamydia* is clearly evident in the study by He *et al.*, (2005). The adoptive transfer of IL-10-deficient DCs pulsed with UV-inactivated EBs, predisposed to developing a Th1-dominant response, reduced the peak of infectious burden by 4 $\log_{10}$ and resolved the infection by day 6 p.i, compared to day 42 p.i for control animals. These DCs were found to express an elevated level of

activation and maturation markers, CD11c, CD40, CD80, CD83 and CD86, and possessed heightened T cell-priming abilities (He *et al.*, 2005). Therefore, DC maturation determines the intensity and longevity of immunity that develops against *Chlamydia*, a factor readily manipulated with modern adjuvants.

Cytosine-phosphate-Guanine-oligodeoxynucleotide

DNA methylation typically occurs on CpG motifs and is vital for cellular development and differentiation in eukaryotes. The host innate immune system has learned to recognise and respond to prokaryotic DNA, which traditionally has low or no methylation of CpG motifs (Sun *et al.*, 1997). Endonuclease-resistant (phosphorothioate-modified) synthetic oligodeoxynucleotide containing unmethylated CpG islands (CpG-ODN) are now used as adjuvants, which imitate and initiate the host response associated with the presence of microbial DNA. The immunomodulatory effects of CpG are well characterised, signalling through the endosomal TLR9 PRR (Wagner, 2004). Toll-like receptor 9 is expressed by keratinocytes, LCs, plasmacytoid DCs (pDCs), myeloid DCs (mDCs), macrophages/monocytes, mast cells and B cells in mice (Wagner, 2004), the majority of which are abundant in the epithelium. As an excellent stimulator of innate immunity in the epithelium, CpG has become one of the most commonly used mucosal adjuvants in vaccine research. There are three structurally distinct classes of synthetic CpGs, D (A), K (B) and C types, of which D and K are most commonly used (Klinman *et al.*, 2007). Only K type CpGs will be discussed in this section as these are more suited to vaccines targeted to the epithelium because their adjuvant activity is not restricted like D type CpGs to cells that express CXCL16 (Gursel *et al.*, 2006). K type CpGs elicit a pro-inflammatory response, producing TNFα and IL-12 in a MyD88-dependent manner (Roman *et al.*, 1997; Jakob *et al.*, 1998), which in turn augments the crucial Th1 response (Chu *et al.*, 1997). Moreover, this adjuvant type also up-regulates antigen-presenting and co-stimulatory molecules MHCII, CD40 and CD86 on APCs (Sparwasser *et al.*, 1998), invaluable in generating long-lived immunity and overcoming mucosal tolerance. In clinical trials, CpG co-administered with hepatitis B, influenza and anthrax vaccines by injection, increased both cell-mediated and antibody responses in human volunteers with no apparent side effects (Klinman *et al.*, 2000; Halperin *et al.*, 2003; Rynkiewicz *et al.*, 2011).

Mucosal application of CpG in animal models induced protection against reproductive and respiratory tract pathogens, by improving antigen-specific Th1 and mucosal antibody responses (Moldoveanu *et al.*, 1998; Berry *et al.*, 2004; Tengvall *et al.*, 2006). This shows the potential benefit of CpGs for vaccine development against mucosal pathogens, but because a chlamydial vaccine also requires a strong induction of mucosal IgA antibodies, CpG is sometimes used in conjunction with a more Th2-polarising mucosal adjuvant, like CT, to further enhance antibody production (Berry *et al.*, 2004; Skelding *et al.*, 2006; Cheng *et al.*, 2009; Finco *et al.*, 2011).

Cholera toxin

Cholera toxin is an enterotoxin secreted by *Vibrio cholera* that can act as a potent mucosal adjuvant. Cholera toxin consists of two subunits, CTA1 (active) and CTB (binding), linked by the CTA2 subunit (Figure 2.6). The adjuvanticity of CT relies on the ability of the holotoxin to bind and exert its activity within the target host cells (Guidry *et al.*, 1997; Agren *et al.*, 1999b). The CTB subunit consists of a pentamer of high-affinity binding molecules that target the monosialoganglioside GM_1 receptor, essential for normal cellular metabolism (Connell, 2007). As GM_1 receptors are expressed on all nucleated cells, CT is a potent stimulator of innate responses. Following binding and internalisation, CT travels through the endoplasmic reticulum where the enzymatically active CTA1 subunit is separated from the CTB subunit by the degradasome and released into the cytosol. The CTA1 subunit catalyses adenosine diphosphate (ADP)-ribosylation of adenylate cyclase leading to an accumulation of cyclic adenosine monophosphate (cAMP). This causes an electrolyte imbalance and rapid efflux of water from the effected cells, which causes the severe diarrhoea commonly associated with a gastrointestinal infection with *V. cholera*. Although the precise immunomodulatory mechanisms behind adjuvanticity are still unknown, CT has been found to increase epithelial permeability, pro-inflammatory cytokine production, IgA class switching and expression of co-stimulatory molecules CD80, CD86 and CD83 by APCs (Bromander *et al.*, 1991; Agren *et al.*, 1997; Cong *et al.*, 1997; Lavelle *et al.*, 2004; Holmgren and Czerkinsky, 2005; Lycke, 2005). Cholera toxin elicits predominantly a Th2 dominant phenotype, although a mixed Th1/Th2 response is also frequently reported (Gagliardi

et al., 2000; Eriksson *et al.*, 2003). Intranasal immunisation with p55gag adjuvanted with CT, induced antigen-specific IgG/IgA and Th1/Th2-type $CD4^+$ cells in vaginal mucosa in non-human primates (Imaoka *et al.*, 1998). A recent human clinical trial reported that TC immunisation with the functionally similar LT, elicited gastric anti-toxoid responses, which resulted in reduced severity of enterotoxigenic *Escherichia coli* (ETEC) associated illnesses (travellers' diarrhoea) without any toxic side effects (McKenzie *et al.*, 2007; Frech *et al.*, 2008). DUKORAL® a licensed orally administered vaccine against *V. cholera* O1, adjuvanted with recombinant CTB, provided 100% protection for 4 – 6 months and was efficacious for the majority of recipients for up to 5 years (Sanchez and Holmgren, 2010). Therefore, enterotoxins and their constituents have proven effective as an adjuvant for needle-free vaccines, by inducing mucosal immunity in animal and human trials.

Cholera toxin/Cytosine-phosphate-Guanine-oligodeoxynucleotide

As protection against *Chlamydia* requires both a Th1 cell-mediated and Th2-driven antibody response, many vaccines have adopted the use of Th1 and Th2 polarising adjuvant combinations The combination of CT and CpG co-administered with the MOMP and delivered via needle-free routes significantly reduced the duration and magnitude of *Chlamydia* infections in the murine respiratory and reproductive tract model (Berry *et al.*, 2004; Skelding *et al.*, 2006; Cheng *et al.*, 2009). Meeting the criteria outlined for an ideal chlamydial vaccine, i.e. providing a balanced Th1 response with a local mucosal *Chlamydia*-specific Th2-driven antibody response capable of reducing likelihood of transmission, CT/CpG plus the MOMP has become our experimental "gold standard" *Chlamydia* vaccine. However, the toxicity of these adjuvants has prevented their approval for human use. Repeated doses of CpG were shown to have a strong correlation with the enhancement of autoimmunity, tissue abnormalities and susceptibility to toxic shock syndrome (Heikenwalder *et al.*, 2004; Klinman *et al.*, 2007; DeFrancesco, 2008). Safety concerns have also arisen over ADP-ribosyltransferase adjuvants delivered via the IN immunisation route, following the withdrawal of Nasalflu® from the market. Nasalflu®, a trivalent influenza vaccine containing the inactivated flu virosome together with the LT adjuvant, induced a high influenza-specific mucosal IgG and IgA titres that was associated with 85-90% protection against infection in humans (Glueck, 2001). Even though the

vaccine was efficacious, the promiscuity of the adjuvant to bind all nucleated cells, including nerve tissues, led to central nervous system (CNS) complications (Bell's palsy) in some patients (Fujihashi *et al.*, 2002; Couch, 2004; Kiyono and Fukuyama, 2004; Mutsch *et al.*, 2004). Cases of Bell's palsy were also reported in phase I clinical trials of IN delivered *M. tuberculosis* and HIV vaccines which also contained the LT adjuvant (Lewis *et al.*, 2009). This is a perfect example of the problems facing needle-free vaccine development and highlights the need to develop either safer ways to deliver these adjuvants or create detoxified derivatives of the problematic yet successful (in animal models) enterotoxins. The potency of mucosal adjuvants, necessary to overcome tolerance toward co-administered antigens, is often too toxic for use in a human prophylactic vaccine. Although, utilisation of different routes of immunisation can limit the toxicity associated with certain adjuvants like CT and CpG (Heikenwalder *et al.*, 2004; Klinman *et al.*, 2007; DeFrancesco, 2008; Lewis *et al.*, 2009), while still harnessing their adjuvanticity. The great success of adjuvants like CT in animal models and human trials has been a major driver for the development of non-toxic, yet immunogenic adjuvant derivatives that can be used in humans for the induction of mucosal immunity.

Cholera toxin A1 subunit-dimer of immunoglobulin binding subunit (CTA1-DD)

Separating the toxicity of CT from the adjuvanticity has proven particularly difficult, as both characteristics are coupled with the binding and enzymatic activity of the holotoxin (Guidry *et al.*, 1997; Agren *et al.*, 1999b). The CTB subunit alone has proven to be a poor adjuvant, consistently inducing antigen-specific tolerance (Sun *et al.*, 1994; Holmgren *et al.*, 2003). Cholera toxin B subunit vaccines have even been used as suppressers of autoimmune diseases (Sun *et al.*, 1994; Sun *et al.*, 1996; Bergerot *et al.*, 1997; Sobel *et al.*, 1998; Sun *et al.*, 2006). Although physical coupling of an antigen to the B subunit can enhance antigen uptake across the mucosal barrier and improve antigen-presentation, CTB and other detoxified mutants of CT that contain the binding subunit, still retain the risk of CNS accumulation following IN immunisation (van Ginkel *et al.*, 2000; Lycke, 2005; van Ginkel *et al.*, 2005). Mutants containing the CTA1 ADP-ribosyltransferase subunit on the other hand, now linked conclusively to immunogenicity (Rappuoli *et al.*, 1999; Hasselberg *et al.*, 2010), have shown more promise. The CTA1-DD adjuvant consists of the

enzymatically active CTA1 subunit of CT, genetically linked to a dimer of an Ig-binding domain (D) from the staphylococcal protein A (Agren *et al.*, 1997). This specifically targets the activity of the CTA1 subunit primarily, but not exclusively (Akhiani *et al.*, 2006), towards B cells opposed to all nucleated cells as with CT (Agren *et al.*, 1997; Agren *et al.*, 1998; Agren *et al.*, 1999a; Agren *et al.*, 1999b; Agren *et al.*, 2000). Encompassing both binding and enzyme activity, CTA1-DD retains the adjuvanticity of the native holotoxin, but most importantly, not its toxicity (Agren *et al.*, 1997; Agren *et al.*, 1999b). Primates and mice can be given >200µg of CTA1-DD without any profound side effects, whereas an equivalent amount of CT is normally lethal in mice (Agren *et al.*, 1997; Agren *et al.*, 1999b; Lycke, 2004; Sundling *et al.*, 2008). CTA1-DD also does not accumulate in nerve tissue following IN immunisation (Eriksson *et al.*, 2004), which makes CTA1-DD an excellent candidate for mucosal vaccine delivered by the IN route.

Although the ADP-ribosylating activity of the CTA1 subunit is crucial, the precise underlying mechanisms behind the adjuvanticity of CTA1-DD, like CT, are still largely unknown. The CTA1-DD adjuvant has been shown to target the membrane-bound B cell receptor (BCR) expressed by naive B cells, complement receptors on follicular dendritic cells (FDC) and potentially bind free Ig to form immune complexes (Figure 2.6) (Agren *et al.*, 1998; Fang *et al.*, 2010; Mattsson *et al.*, 2011). Binding to membrane bound Ig, CTA1-DD up-regulates expression of CD86 and CD80 on B cells (Agren *et al.*, 1997), which is needed for adequate priming of T cells and to overcome mucosal tolerance. The CTA1-DD adjuvant also has an anti-apoptotic affect on B cells that stimulates germinal centre (GC) formation, responsible for IgA class switching recombination (CSR), generating long-lived memory B cells and antibody-secreting plasma cells (Agren *et al.*, 1997; Agren *et al.*, 1999b; Bemark *et al.*, 2011). Both binding and enzymatic activity are critical for stimulation of B cells (Agren *et al.*, 1999b). Interestingly, it was found that increased cAMP levels, suggested to be critically linked with the immunogenicity of CT, were not elevated in B cells treated with CTA1-DD (Agren *et al.*, 1998). Moreover, stimulation of the anti-apoptotic B cell factor Bcl-2 by CTA1-DD occurred in athymic mice, contrary to the holotoxin that failed to prevent apoptosis in the absence of T cells (Agren *et al.*, 2000). Despite differing from CT only by binding

ability, CTA1-DD appears to have an entirely different mechanism of action on B cells.

Even though CTA1-DD was designed to target B cells, its immunogenicity is partially retained in μMT mice (B cell-deficient) (Akhiani *et al.*, 2006). This suggests the adjuvanticity of CTA1-DD is primarily B cell-dependent, but B cell-independent mechanisms may also be involved (Figure 2.6). Capable of binding to both Fc and Fab antibody fragments, CTA1-DD may cross-link free Ig to form immune complexes. Pre-incubation of CTA1-DD with polyclonal IgG induced greater degranulation and TNFα production by mast cells than CTA1-DD alone, which was attributed to the formation of immune complexes and cross-linking of Fc receptors (Fang *et al.*, 2010). Production of TNFα could be detected in all controls containing the Ig-binding domain, implicating the DD motif and not the CTA1 subunit in this stimulatory response. However, the immune complex does not appear to develop *in vivo* following intravenous (IV) or intraperitoneal (IP) administration CTA1-DD (Agren *et al.*, 2000). Furthermore, FcγR-deficient mice exhibited an unaltered antigen-specific response to CTA1-DD-adjuvanted vaccines (Agren *et al.*, 2000; Akhiani *et al.*, 2006), suggesting that the antibody complex may be an *in vitro* artefact and not contribute to the immunogenicity of CTA1-DD *in vivo*.

More recently, CTA1-DD was also found to bind to follicular dendritic cells (FDC) in B cell follicles, not by the immune complex FcR mechanism, but in a CR2-dependent manner (Mattsson *et al.*, 2011). Intravenous (IV) immunisation with CTA1-DD enhanced the formation of GCs, antibody production and T cell-dependent responses by activation of the complement pathway, a function abrogated in $CR2^{-/-}$ mice. This action was dependent on the both binding and enzyme motifs. Interestingly, enhancement of antibody production was severely impaired in CR2-deficient mice given CTA1-DD intranasally, which may indicate that this function could also be activated after mucosal immunisation.

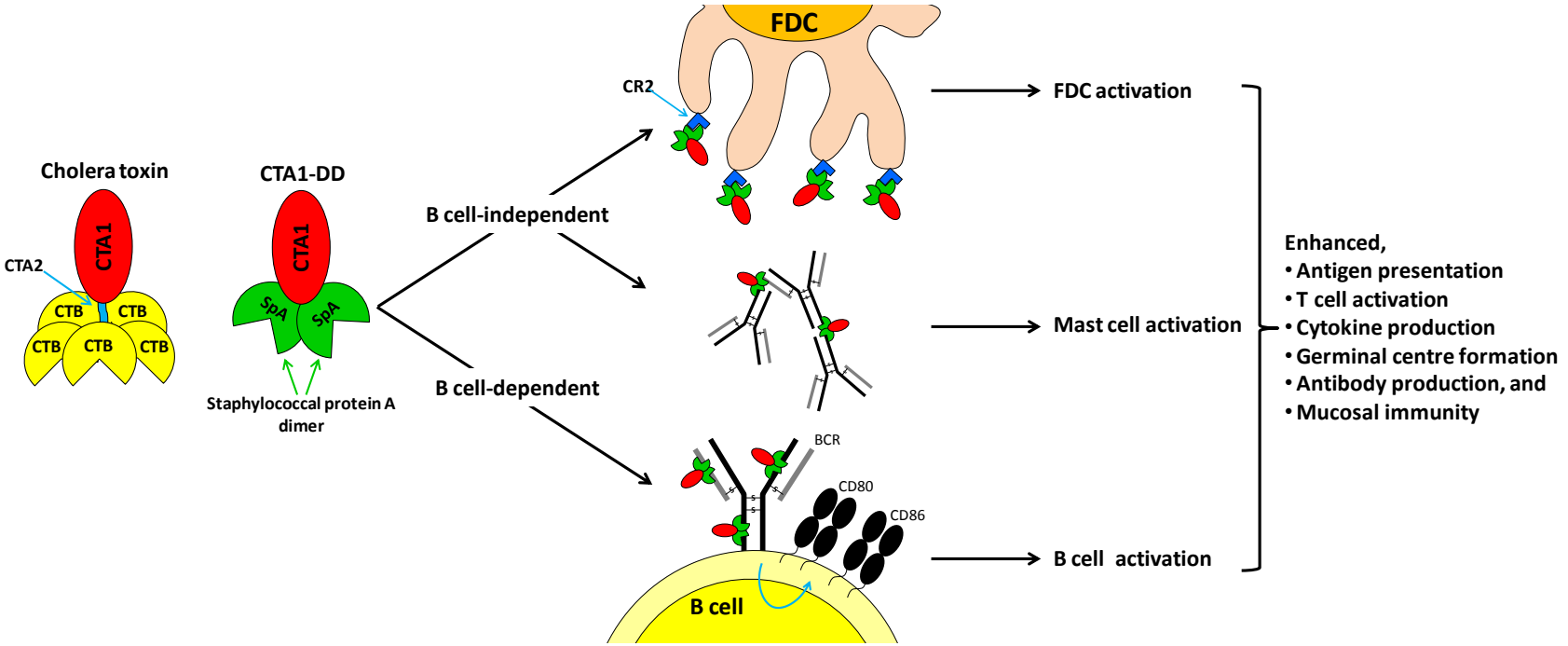


Figure 2.6: Illustration of the current published and theorized mechanisms behind the adjuvanticity of CTA1-DD.

The adjuvant function of CTA1-DD, consisting of the CTA1 subunit of CT bound to a dimer of staphylococcal Ig-binding proteins, involves B cell-dependent and -independent mechanisms. The B cell-dependent mechanism entails binding of membrane bound Ig on B cells, which promotes the expression of co-stimulatory molecules CD80 and CD86. B cell-independent mechanisms involves the activation of FDCs by complement receptor CR2 and the formation of the immune complex by cross-linking free antibodies, which may subsequently activate mast cells. CTA1-DD is known to promote mucosal immunity by enhancing antigen presentation, T cell activation, cytokine production, GC formation and antibody production.

Mucosal delivery of CTA1-DD has been shown to promote antibody and cell-mediated responses. The CTA1-DD adjuvant invokes a balanced Th1/Th2 response (Eriksson and Lycke, 2003), essential in developing protective immunity against *Chlamydia*. Trialled experimentally in research against HIV (Sundling *et al.*, 2008), Influenza A (De Filette *et al.*, 2006), *Helicobacter pylori* (Akhiani *et al.*, 2006), *Mycobacterium tuberculosis* (Andersen *et al.*, 2007) and Rotavirus (Choi *et al.*, 2002; McNeal *et al.*, 2007), CTA1-DD improved mucosal immune response in all studies and protection in models of infectious challenge. A pilot study by Cunningham *et al*., (2009), assessed the ability of IN immunisation with the MOMP co-administered with or without CTA1-DD to promote mucosal immunity. The animals receiving the MOMP plus CTA1-DD produced significantly higher systemic and mucosal *Chlamydia*-neutralising antibodies than the antigen control (Cunningham *et al.*, 2009), a responses conclusively linked with improved clearance and reduced pathology *in vivo* (Morrison *et al.*, 1995; Su *et al.*, 1997; Yang and Brunham, 1998; Murthy *et al.*, 2004; Morrison and Morrison, 2005; Rodriguez *et al.*, 2006). Interestingly, the levels of MOMP-specific antibodies, as determined by enzyme-linked immunosorbent assay (ELISA), were remarkably similar with and without the addition of the CTA1-DD. It was suggested that direct interaction between the adjuvant and B cells may have influenced antigen recognition or epitope selection leading to higher neutralising antibodies and a reduced level of chlamydial shedding following an IVag challenge. This particular study has provided valuable insight into the immune-mediating capability of CTA1-DD, its potential to substitute toxic mucosal adjuvants in a novel *Chlamydia* vaccine.

SUMMARY

A vaccine is needed to control the chlamydial pandemic. Thus far, there has been limited progress towards the development of an effective human vaccine against *Chlamydia*, partly due to an inability of current adjuvants to stimulate mucosal immunity without compromising safety. This study assessed the ability of the novel non-toxic mucosal adjuvant CTA1-DD to stimulate mucosal immunity, by direct comparison to the proven yet toxic CT/CpG. Different needle-free immunisation routes were also utilised not only to stimulate the CMIS, but to potentially lessen the

toxicity CT/CpG without affecting immunogenicity. Protection against *Chlamydia* genital and respiratory tract infections were used to evaluate each vaccines efficacy and possibility to benefit the human population.

CHAPTER THREE: GENERAL MATERIALS AND METHODS

GENERAL REAGENTS AND CONSUMABLES

Concentrated powder and liquid chemicals, where not specified, were obtained from Merck Pty Ltd, Kilsyth, Australia. Consumables, such as disposable tubes and pipette tips were obtained from Lab Advantage, Murarrie, Australia unless otherwise stated.

CHLAMYDIA STRAINS AND CELL LINES

Cell lines

The McCoy mouse fibroblast cell line was used for propagation and quantification of *C. muridarum*. Original stocks were purchased from the American Type Culture Collection (ATCC, Virginia, USA). McCoy cell stocks were cryopreserved in media containing 10%v/v dimethyl sulfoxide (DMSO) (Sigma-Aldrich Pty Ltd, Castle Hill, Australia) and continually checked by polymerase chain reaction (PCR) for *Mycoplasma* contamination.

Chlamydia strain

C. muridarum Weiss strain VR-123, formerly mouse pneumonitis biovar of *C. trachomatis* (MoPn), was obtained from the ATCC.

CELL CULTURE SOLUTIONS

Phosphate buffered saline (PBS)

Phosphate buffered saline was prepared by dissolving 8g of NaCl, 0.2g of KCl, 1.44g of $NaHPO_4$ and 0.24g of KH_2PO_4 in 1L of double distilled water (ddH_2O) (Millipore MilliQ water purification system – Millipore Australia Pty Ltd, Australia) and pH adjusted to 7.4 using NaOH.

Fetal calf serum (FCS)

Fetal calf serum (Lonza Australia Pty Ltd, Mt Waverley, Australia) was heat-inactivated by placing the bottle in a 56°C water bath for 45 min, to ensure destruction of protease and complement.

Cell culture growth medium

McCoy cells and *C. muridarum* we supplemented complete Dulbecco's minimal essential medium (DMEM) containing 5% FCS, 4mM L-glutamine, 50µg/ml gentamycin (Invitrogen, Mt Waverley, Australia) and 100µg/ml streptomycin sulphate (Sigma-Aldrich).

Trypsin

A 10X solution of trypsin ethylenediaminetetraacetic acid (EDTA).4Na (Invitrogen) was diluted to 2X with sterile PBS before use.

Sucrose phosphate glutamine buffer (SPG)

Sterile SPG was used as a cryopreservative for storage of all chlamydial stocks and samples. SPG was prepared to contain a final concentration of 219mM sucrose, 3.8mM KH_2PO_4, 8.6mM Na_2HPO_4 and 4.9mM glutamic acid, dissolved in ddH_2O. The pH was adjusted to 7.0 before the sterilising by autoclaving.

CHLAMYDIA CELL CULTURE

Growth and maintenance of McCoy cells

McCoy cell were continually passaged in 175cm^2 tissue culture flasks (BD Bioscience, North Ryde, Australia) in complete DMEM in a 37°C humidified incubated with 5% CO_2 until 100% confluent. All reagents were pre-warmed to 37°C in a water bath prior to use. Spent media was discarded into a waste bottle containing 12.5%w/v sodium hypochlorite. The monolayer was subsequently washed with sterile PBS and discarded into the waste container before adding 3mL of trypsin to the flask. The flask was sealed and placed into the incubator for 2 min. To facilitate cell detachment, the sides of the flask were gentle tapped. Once cells were dislodged, 5mL of complete DMEM was added to the flask to inhibit tryptic activity. This cell suspension was then split over the required amount of flasks, supplemented with a further volume of media and returned to the incubator.

Propagation of *Chlamydia*

Samples of *C. muridarum* EBs obtained from the ATCC were subsequently propagated in our laboratory. A frozen culture flask, infected with *C. muridarum* and stored in SPG, was removed from the -80°C and partially thawed in the incubator. Sterile glass bead were added to the flask and the infected cells were sonicated in bath sonicator (Cole-Parmer 8891 Ultrasonic cleaner – John Morris Scientific, Eagle Farm, Australia) for 10 min. The media was then transferred to a 50mL tube and centrifuged at 4°C for 5 min at 500x*g* to remove insoluble cellular debris. The supernatant was transferred to a fresh tube and either stored at -80°C or used to infect an 80% confluent flask of McCoy cells. As a general rule, no more than one eighth of the supernatant was used to infect a subsequent flask. Two – 4 hr after infection, host cell protein synthesis was inhibited by replacing the media in the flask to media containing 1µg/mL of cycloheximide (Invitrogen). The infected cells were then incubated in a humidified incubator at 37°C with 5% CO_2 for between 24 – 30 hr, or until active inclusions containing EBs could be visualised under an Olympus CK2 inverted light microscope (Olympus Australia Pty Ltd, Mt Waverley, Australia). Infection was stopped by adding an equal volume of SPG to the media then freezing at -80°C. Frozen flasks were used for further propagation of *Chlamydia*.

Ultra-purification of *Chlamydia*

Frozen flasks were defrosted and sonicated as describe above. *Chlamydia* was semi-purified from the infected supernatants by centrifuging at 20,000x*g* for 20 min at 4°C. The subsequent pellet was resuspended in media containing 500U/mL heparin sulphate (Sigma-Aldrich), 10mM $MgCl_2$ and 40U/mL DNase I (Roche, Castle Hill, Australia) and incubated at 37°C for 30 min. *Chlamydia* was pelleted at 20,000x*g* and the bacterial pellet was washed repeatedly and resuspended in ice-cold SPG. This solution was then layered over 29%v/v urografin Ultravist® (Sigma-Aldrich) and spun at 70,000x*g* for 30 min in a Optima® L-90K ultracentrifuge (Beckman Coulter, Gladsville, Australia). The chlamydial pellet was washed repeatedly and resuspended in SPG, then layered over a gradient column containing 40%, 34% and 29% urografin and again spun at 70,000x*g* for 30 min. Chlamydial EBs were

removed from the interface between the 34% and 40% urografin layers, washed, then stored in small aliquots of SPG and frozen down at -80°C.

Quantification of *Chlamydia*

Using a confluent flask of McCoy cells, cells were detached and counted with a haemocytometer using 0.4%w/v trypan blue (Sigma-Aldrich) as a live/dead cell discriminator. The cell suspension was diluted to a final concentration of 1 x 10^5 cells/mL and 500µL containing 5 x 10^4 cells was placed into each well of a 48-well plate (BD Bioscience) and incubated overnight at 37°C in a humidified incubator. The concentration of *Chlamydia* in the frozen stocks was determined by serial dilution of a known volume of the original stock onto the 80% confluent monolayer. *Chlamydia* was propagated as described above before stopping the infection by fixation and staining with a *Chlamydia*-specific antibody (see below).

Sheep anti-*C. muridarum* polyclonal serum

Sheep anti-*Chlamydia* serum was ordered from the Institute of Medical and Veterinary Science (IMVS), Dulwich, Australia. A Merino/Border Leicester x-bred wether sheep, was immunised SC with 1mg of recombinant *C. muridarum* MOMP in complete Freunds adjuvant, repeated every 3 – 5 weeks a total of three times in incomplete Freunds. The sheep was bled 3 weeks after the final boost. The blood was allowed to clot at 4°C for overnight, after which the serum was separated from whole blood by centrifugation. Murine cross-reactive antibodies were removed from the sheep anti-serum following incubation at 4˚C for 1 hr with 10^7 BALB/c splenocytes/mL of serum. Aliquots of adsorbed serum were stored at -80°C.

Fixing an staining for *Chlamydia*

Following propagation and the visualisation of active inclusions, the media was removed from the wells and washed with PBS. The infected monolayer was then fixed with 100% methanol for 10 min then subsequently washed with PBS. In preparation for *Chlamydia*-specific staining, non-specific binding was blocked overnight at 4°C with a blocking solution containing 5%v/v FCS, 0.05%w/v sodium azide in PBST (0.05%v/v Tween20). Cells were then incubated with a 1/500 dilution of the *Chlamydia*-specific sheep anti-serum in blocking solution for 1 hr at 37°C.

Rinsed thoroughly with PBST following incubation, cells were then treated with blocking solution containing 1µg/mL of donkey anti-sheep AlexaFluor-488 secondary (Invitrogen) and 10ng/mL 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen) for 1 hr at 37°C. Cells were then rinsed thoroughly with PBST and stored in PBS at 4˚C in the dark. Images from 4 random fields of view (FOV) were taken from each well using a Nikon Eclipse TE2000-U fluorescent inverted microscope with Nikon Digital Eclipse DXM 1200C camera (Nikon Pty Ltd, Lidcombe, Australia). Chlamydial inclusion-forming units (IFU) were counted using MetaMorph Imaging Series 7.6 software (Molecular Devices, Sunnyvale, USA) based on fluorescent pixel area and intensity above background. Quantification of chlamydial IFU/well was calculated by multiplying the number of IFU/FOV by the FOV/well. Quantification of chlamydial IFU/mL was calculated by multiplying the number of IFU/well by 1000 and dividing by the volume aliquoted into each well in µL.

RECOMBINANT MOMP PREPARATION

Native MOMP (nMOMP), isolated directly from chlamydial EBs, has been shown to elicit a protective response equivalent to that induced following a live infection (Pal *et al.*, 2005; Sun *et al.*, 2009). Recombinant MOMP (MOMP), closely mimicking conformational epitopes of nMOMP, can also induce a similar level of protection without the larger cost associated with production (Sun *et al.*, 2009).

Luria-Bertani (LB) broth and agar

Luria-Bertani broth was prepared by dissolving 10g of tryptone, 5g of yeast extract (Oxiod, Australia Pty Ltd, Thebarton, Australia) and 10g of NaCl in 1L of ddH_2O. Luria Bertani agar contained of LB broth with the addition of 15g of agar (Oxoid). Each solution was sterilised by autoclaving at 121°C for 15 min.

Bacterial clone

Recombinant *C. muridarum* MOMP was purified from the *E. coli* clone (DH5α[pMMM3]), transformed with the pMAL-c2 ampicillin-resistant vector encoding the recombinant maltose-binding protein (MBP) fusion protein (a generous

gift from Harlan Caldwell, Rocky Mountain Laboratories) (Su *et al.*, 1996). Clones were stored in LB broth containing 80%w/v glycerol at -80°C.

Large scale recombinant protein expression and purification

A single colony of DH5α[pMMM3] was isolated from a glycerol stock using the sixteen streak method on LB agar plates containing 100µg/µL of ampicillin (Roche) and then incubated at 37°C overnight. A 15mL overnight starter culture grown from a single colony was used to infect a 1L within a 2L conical flask. This large scale culture was incubated at 37°C under 220rpm rotation until the turbidity of the media reached optical density (OD) of 0.4, measured at a wavelength of 400nm using a DU800 spectrophotometer (Beckman Coulter). Expression of the recombinant protein was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich Pty Ltd, Castle Hill, Australia) to the culture at a final concentration of 0.15mM and incubating for a further 2 hr. The bacterial pellet was sonicated (Ultrasonic liquid processor, Misonix, Farmingdale, USA) using spun at 4,000x*g* to pellet the insoluble inclusion bodies containing the MOMP protein. The inclusion bodies were solubilised using 8M urea containing a bacterial protease inhibitor cocktail (Cat No. P8465, Sigma-Aldrich) and incubated for a further 2 hr at 37°C under 100rpm rotation. The soluble MOMP protein fraction was then dialysed in a stepwise manner, using a decreasing gradient of urea until eventually equilibrated against PBS. The refolded MOMP-MBP (81kDa, 39kDa and 41kDa, respectively) (Su *et al.*, 1996) preparation was then further purified and concentrated by size exclusion using the Centriprep® centrifugal filter unit, Ultracel-50 membrane 50kDa molecular weight cut-off (MWCO) column (Millipore Australia Pty Ltd, North Ryde, Australia). The purified MOMP was quantified with the BCA Protein Assay® kit (Thermo Fischer Scientific, Rockford, Illinois, USA)) and visualised by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). To maintain solubility and a more natural conformation of the MOMP, MBP was not cleaved from the purified protein (Su *et al.*, 1996). Immunoreactivity of the MOMP was assessed against samples from a natural infection and hence the native MOMP conformation, using western blotting and lymphocyte proliferation assays. The final product was concentrated to 20mg/mL and stored at -80°C in aliquots to prevent repeated freeze thawing.

Lipopolysaccharide removal and quantification

Lipopolysaccharide was quantified using ToxinSensor® chromogenic *Limulus* amebocyte lystate (LAL) endotoxin assay kit (Cat No. L00350, Genscript Inc, Pistcataway, New Jersey, USA) as per the manufacturer's instructions. LPS contamination was removed by diluting the concentrated MOMP preparation to 0.5mg/mL with sodium deoxycholate (Sigma-Aldrich) to the critical micelle concentration of 0.1%w/v (Jang *et al.*, 2009), then concentrating again by size exclusion using the centrifugal filtration. This process was repeated until LPS was reduced to quantities below 0.1µg/dose (Eriksson *et al.*, 2004). Residual sodium deoxycholate was removed by dialysis against PBS.

ANIMALS HOUSING

Ethics statement

All studies and procedures were approved by the QUT Animal Ethics Committee (approval number 0800000432).

Mice

Female BALB/c mice were sourced from the Animal Resource Centre (ARC) (Canningvale, Western Australia) at 6 weeks of age. Five animals were used in each group unless otherwise stated. Animals were given food and water ad libitum and maintained in a temperature controlled environmental with a cycle of 12 hr of light and 12 hr of darkness. Animals were euthanised humanly by IP injection with Lethabarb® (200 mg/kg) (Virbac Animal Health, Reagents Park, Australia).

ADJUVANTS

Cholera toxin A1 subunit-dimer of immunoglobulin binding subunit

The CTA1-DD adjuvant was a generous gift from Nils Lycke at MIVAC, Gothenburg, Sweden. The CTA1-DD adjuvant was purified as previously described (Agren *et al.*, 1997) and stored in PBS at -80°C.

Cholera toxin

Lyophilised CT (List Biological Laboratories, Sapphire Bioscience Pty Ltd, Redfern, Australia) was resuspended in PBS and stored at 4°C.

Cytosine-phosphate-Guanine-oligodeoxynucleotide 1826

The CpG-ODN 1826[c] adjuvant (5′-TCC ATG ACG TTC CTG ACG TT-3′) (Sigma-Aldrich) was made on a nuclease-resistant phosphothioate backbone (Jakob *et al.*, 1998). Lyophilised CpG-ODN was resuspended in PBS and stored at -20°C.

IMMUNISATION

Mice were immunised on days 0, 7, 14 and 28 as described below. This immunisation schedule was chosen because it will allow a retrospective comparison between past and present work (Berry *et al.*, 2004; Hickey *et al.*, 2005; Skelding *et al.*, 2006; Cunningham *et al.*, 2009; Hickey *et al.*, 2009). Each route included antigen (MOMP), adjuvant (CTA1-DD and CT/CpG-ODN) and unimmunised (PBS) control groups. All vaccines were made fresh before each immunisation.

Intranasal immunisation

Animals in the IN group were lightly anesthetised with 4%v/v isofluorane by inhalation (Abbott Australasia Pty Ltd, Botany, Australia). The anesthetised mice were placed on their backs, held at a downward angle (Figure 3.1) then immunised with the MOMP (100µg) and either CTA1-DD (20µg) (MIVAC) or CT (List Biological Laboratories) (5µg)/CpG-ODN 1826[c] (Sigma-Aldrich) (10µg) mixed in a 10µl volume, 5µl applied to each nare.

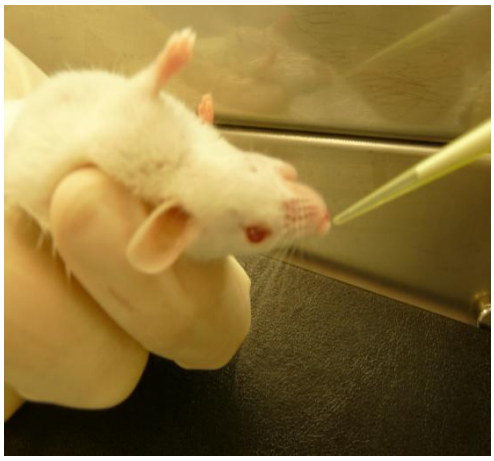


Figure 3.1: Photo of IN immunisation.
The mouse was firstly anesthetised with isofluorane and then held on its back at a downward angle. The vaccine was administered slowly to each nare in a volume totalling no more than 10µL. The mouse was then placed back in the cage to recover completely.

Transcutaneous immunisation

Animals in the TC group were heavily sedated with an IP injection with ketamine (Parnell Laboratory Pty Ltd, Alexandria, Australia) (100mg/Kg) and xylazine (Bayer Australia Ltd, Pymble, Australia) (10mg/Kg). A 1.5cm^2 space on the backs of mice at the base of the tail was then shaved using clippers, with care taken not to break the skin. The skin was pre-treated first with acetone, then with a solution containing dodecylpyridinium chloride (DPC) (0.33%w/v), isopropyl myristate (IPM) (0.33%w/v) and methyl pyrolidone (MPR) (0.33%w/v) (Sigma-Aldrich) (Karande *et al.*, 2009) and finally rehydrated with copious amounts of PBS. Each permeation and rehydration step was applied directly to the skin with a transfer pipette individually. Each step was also accompanied by a vigorous yet gentle abrasion of the skin with a piece of paper towel doused in the same reagent. Mice then received GM-CSF (12.5ng) (Hickey *et al.*, 2005) with the MOMP (200µg) and either CTA1-DD (20µg) or CT (10µg)/CpG-ODN 1826^c (10µg) mixed in a volume of 50µL. The immunisation was contained for a 24 hr time period using a patch system consisting of gauze, Opisite Flexifix® (Smith & Nephew Australia, Mt Waverley, Australia) and Micropore® surgical tape (3M Australia, North Ryde, Australia) (Figure 3.2). This immunisation protocol was modified and improved from previous studies (Berry *et al.*, 2004; Hickey *et al.*, 2005) (Appendix 1)

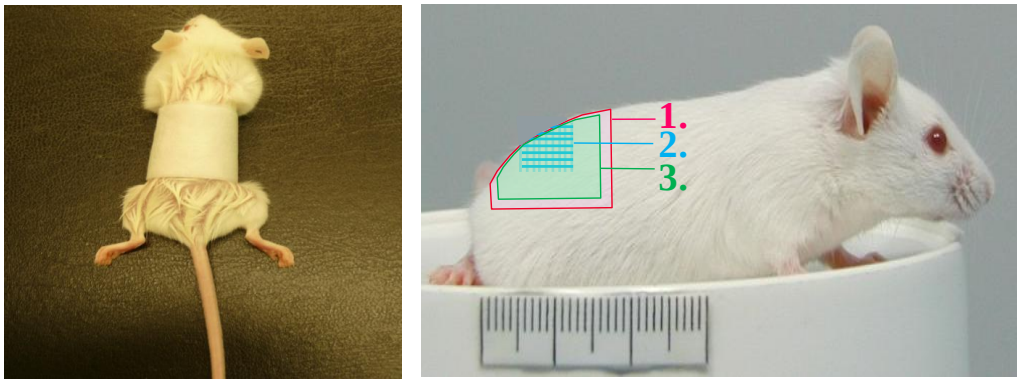


Figure 3.2: Photo of TC immunisation and diagram of the patch system.
Assemble of the patch system (right picture) began by (1) shaving the lower back of the anesthetised mouse above the tail. The shaved area was then permeabilised using acetone and DPC, IPM, MPR. The permeabilised skin was then washed repeatedly with PBS in preparation for the vaccine. (2) The vaccine was spread over a small area of the permeabilised skin and covered with gauze. (3) The vaccine was retained on the skin with a waterproof seal of Opisite Flexifix®. The patch was then secured to the mouse using surgical tape (left picture). The mouse was then placed back in the cage to recover completely.

Sublingual immunisation

The SL group was also heavily sedated with ketamine and xylazine. The MOMP (100µg) was mixed with either CTA1-DD (20µg) or CT (5µg)/CpG-ODN 1826[c] (10µg) in a 7µL volume was applied directly to the ventral side of the tongue (Figure 3.3) and left for 1 hr with the head of the mice maintained in ante-flexion (Cuburu *et al.*, 2007). Antigen dose curve was optimised for SL immunisation for the MOMP antigen using antigen-specific proliferation and serum IgG (Appendix 2).

Figure 3.3: Photo of SL immunisation.
The mouse was firstly anesthetised and placed in a position (ante-flexion) to prevent the vaccine entering the gastrointestinal tract. The vaccine was administered underneath the tongue (picture), in a volume no greater than 7µL. The mouse was then placed back in the cage to recover completely.

Statistical analysis

All data was presented as the mean ± standard deviation (SD). All statistics were performed using GraphPad Prism® version 5.00 (GraphPad Software Inc, California, USA). Significant differences were determined using a one-way analysis of variance (ANOVA) with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

CHAPTER FOUR: ANTIGEN-SPECIFIC SYSTEMIC AND MUCOSAL IMMUNE RESPONSES FOLLOWING VACCINATION

INTRODUCTION

Animal and human studies have indicated that $CD4^+$ Th cells, predominantly those secreting IFNγ, are essential for protection against chlamydial infection and disease. $CD4^+$ Th1 cells target chlamydial RBs undergoing active replication by activating the infected host cell's defences against intracellular pathogens (Cotter *et al.*, 1997b). This response restricts the growth and hence the amount of *Chlamydia* shed into the lumen and available for transmission (Perry *et al.*, 1997). However, $CD4^+$ Th1 cells are unable to directly prevent extracellular *Chlamydia* from infecting new host cells during the establishment of infection. Protection against attachment of chlamydial EBs and the spread of infection to a new host or cell requires an antibody response. Antibodies can neutralise extracellular *Chlamydia* by directly preventing attachment or by opsonising EBs for engulfment by phagocytes (Moore *et al.*, 2002). This form of protection is predominantly responsible for raising the infectious threshold necessary to establish an infection (Su *et al.*, 1997; Williams *et al.*, 1997). Therefore, due to the unique biphasic chlamydial lifecycle, containing both extracellular and intracellular forms, an effective vaccine against *Chlamydia* needs to elicit a Th1-driven cell-mediated as well as Th2-driven humoral antibody response. Furthermore, the effectiveness of a vaccine can be enhanced further if the *Chlamydia*-specific T and B cell responses are localised to or capable of being rapidly recruited to the anatomical portal of entry for the invading pathogen, in what is known as a mucosal response.

Parenteral vaccines can successfully prevent infections caused by invasive mucosal pathogens by inducing neutralising antibodies. These vaccines are relatively poor inducers of localised mucosal responses (Muszkat *et al.*, 2000; Holmgren and Czerkinsky, 2005; Cong *et al.*, 2007; Pedersen *et al.*, 2011), which are necessary for protection against pathogens such as *Chlamydia*, which enter through and are predominantly confined to the epithelium. Targeting vaccination to the epithelium more effectively generates mucosal immunity, yet such vaccines often require a powerful adjuvant to overcome the induction of tolerance that often occurs following mucosal exposure to an antigen (Smith *et al.*, 2012). Whilst immunologically effective in animal models, the use of potent adjuvants like the GM_1-ADP

ribosylating toxins (CT or LT) or TLR agonists can be problematic in humans due to inherent toxicity (Heikenwalder *et al.*, 2004; Mutsch *et al.*, 2004). However, the great success of these adjuvants in animal models has been a major driver for the development of non-toxic, yet immunogenic adjuvant derivatives that can be used safely in humans for the induction of both systemic and mucosal immunity.

The CTA1-DD adjuvant is an example of such an agent, derived from the native CT holotoxin. The CTA1-DD adjuvant consists of the enzymatically active CTA1 subunit of CT, genetically linked to a dimer of an Ig-binding domain (Agren *et al.*, 1997). Inclusion of the DD moiety targets immunogenic activity primarily to Ig-expressing B cells (Eriksson *et al.*, 2004; De Filette *et al.*, 2006). The CTA1-DD adjuvant retains the adjuvanticity of CT, inducing robust cell-mediated and mucosal antibody responses (Sundling *et al.*, 2008), but most importantly is up to 1000-fold less toxic in primates than the native holotoxin (Lycke, 2004; Sundling *et al.*, 2008). In addition to the potential for clinical safety (Sundling *et al.*, 2008), the success of CTA1-DD in enhancing mucosal immune responses to mucosal vaccines suggests that CTA1-DD may be an ideal adjuvant candidate for a human chlamydial vaccine (Akhiani *et al.*, 2006; De Filette *et al.*, 2006; Andersen *et al.*, 2007; McNeal *et al.*, 2007; Sundling *et al.*, 2008; Cunningham *et al.*, 2009).

The route a vaccine is administered influences the localisation of the immune response (respiratory and/or genital tract) via the CMIS. The immunisation route chosen can also effectively limit the toxicity of certain adjuvants, while still retaining their immunogenicity. Cholera toxin and CpG are excellent mucosal adjuvants, capable of manipulating innate responses for the induction of robust and long-lived IFNγ-secreting $CD4^+$ T cells and mucosal IgA (Berry *et al.*, 2004; Skelding *et al.*, 2006). Their inherent toxicity however, restricts their use in humans via particular routes. For example, the potent enterotoxin adjuvants delivered via the IN route can bind and accumulate in the CNS with harmful neurological affects and when delivered by the oral route cause diarrhoeal disease. Enterotoxins applied topically however, have been shown to induce a similar immune response to that observed following nasal delivery (Skelding *et al.*, 2006) without the toxic side effects. Recent human clinical trials employing skin delivery of LT, elicited gastric anti-toxoid

responses, which resulted in reduced severity of travellers' diarrhoea (McKenzie *et al.*, 2007; Frech *et al.*, 2008). Importantly, neither study reported any adverse events.

Here we compared two adjuvants, CTA1-DD and the CT/CpG combination, together with the MOMP. Animals were vaccinated via needle-free IN, SL or TC immunisation routes, to induce mucosal responses and limit adjuvant toxicity. In this chapter, we measured the induction of antigen-specific systemic and mucosal immune T and B cell responses in the genital and respiratory tracts following vaccination.

MATERIALS AND METHODS

Timeline

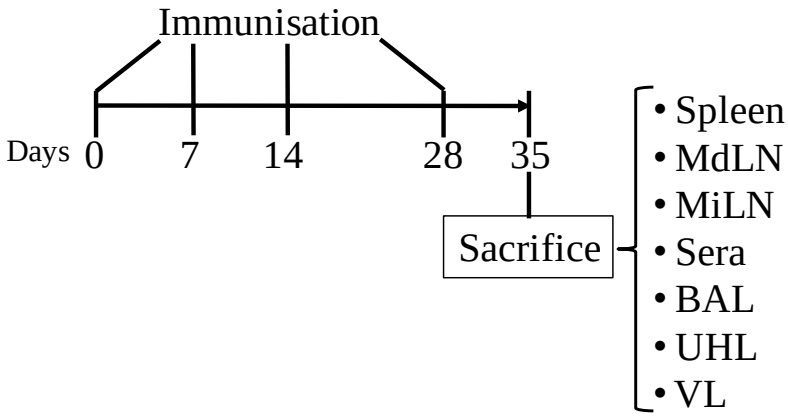


Figure 4.1: Experimental timeline for assessment of systemic and mucosal immune responses.

Mice were immunised on days 0, 7, 14 and 28 and culled 7 days after the final boost. Samples collected included the spleen, mediastinal lymph node (MdLN), medial iliac lymph node (MiLN), serum, bronchoalveolar lavage (BAL), uterine horn lavage (UHL) and vaginal lavage (VL).

Vaccination toxicity

The health of each animal was monitored daily following vaccination according to a strict set of criteria approved by the QUT Animal Ethics Committee. Animals were scored on criteria based on their overall appearance, i.e. weight, coat condition, movement and social interaction. Animals exhibiting multiple signs of distress were examined by a veterinarian and euthanised immediately.

Sample collection

The spleen, MdLN and MiLN (also known as the caudal and lumbar lymph nodes) (Hummel *et al.*, 1986; Van den Broeck *et al.*, 2006) were excised and pooled with their respective groups in complete DMEM. Blood was taken via cardiac puncture, allowed to clot at 4°C, after which the serum was collected by centrifugation. The BAL was collected by flushing the lungs once with 800µl of PBS. The VL was taken by flushing the vagina with 40µl of PBS collected once a day over the 4 day estrous cycle and then pooled for each animal. The UHL was collected by first excising the

tissue, then flushing each uterine horn with 100µl of PBS. Serum and lavage samples were stored at -80°C until required.

In vitro stimulation and proliferation analysis

The spleens and lymph nodes were mechanically disrupted by passing each tissue through a 75µm nylon filter (BD Bioscience). The single cell suspensions were then centrifuged at 500x*g* for 5 min. Each cell pellet was then resuspended in filter-sterilised red blood cell lysis buffer containing 155mM NH_4Cl, 12mM $NaHCO_3$, 100µM EDTA dissolved in ddH_2O and adjusted to pH 7.35 for 5 min on ice. Following incubation the reaction was then stopped using two times the volume of complete DMEM. Cells were centrifuged again at 500x*g* for 5 min and washed a total of two times with Hank's buffered salt solution (HBSS) (Invitrogen) and finally resuspended in complete DMEM containing 50µM β-mercaptoethanol (Sigma-Aldrich). Lymphocytes from each tissue were diluted to 5 x 10^6 cells/mL and 100µL of the cell suspension was seeded into a U-bottom 96-well plate (5 x 10^5 cells/well) in triplicate. Lymphocytes were then supplemented with 100µL normal media (unstimulated) or stimulated with media containing either the MOMP (10µg/well) or *concanavalin A* (0.5µg/well) (data not shown) (Sigma-Aldrich) for 72 hr at 37°C with 5% CO_2 in a humidified incubator. Following incubation 100µL of the media supplementing the stimulated cells was removed and stored at -80°C for analysis of antigen-specific cytokine production. Cells were then incubated with 0.5µCi ^{3}H-thymidine/well at 37°C with 5% CO_2 in a humidified incubator over a 14 hr time period. Following incubation, cells were transferred to glass fibre filter paper (PerkinElmer, Massachusetts, USA) using Brandel 96 Harvester (Brandel, Gaithersburg, USA). The glass filter paper was dried and placed in a plastic sleeve before adding approximately 5mL of MicoBeta scintillation liquid (PerkinElmer). The filter paper was sealed in the plastic sleave and ionising radiation was detected using the Wallac 1450-030 MicroBeta TriLux Liquid Scintillation and Luminescence Counter (PerkinElmer). Cell proliferation was determined by the incorporation of ^{3}H-thymidine into newly synthesised DNA. Results were expressed as a stimulation index, dividing counts per minute (cpm) of the MOMP stimulated by the cpm of the media background.

Measurement of cytokine secretions

Pooled supernatant from stimulated immune cell preparations were analysed for levels of IFNγ, IL-4, IL-10 and IL-17 using a customised BioPlex Mouse Express Pro Cytokine Assay 4-plex (Cat No. M60-009RDPD, Biorad Laboratories, Hercules, California) according to the manufacturer's instructions and read on a Bioplex-200 system with Luminex xMap® technology (Biorad). The levels of TNFα were also detected from the pooled supernatant using the DuoSet ELISA development system (Cat No. DY410, R&D systems – Sapphire Bioscience Pty Ltd, Waterloo, Australia) according the manufacturer's instructions. Both methods used a standard curve to quantify cytokine concentrations.

Antigen-specific antibodies

Individual 96-well ELISA plates (Greiner Bio One, Kremsunster, Austria) were coated with recombinant MOMP. Plates were incubated overnight with 2µg/well of the MOMP in 50µL of borate buffered saline (BBS) containing 0.5M Boric acid, 1.5M NaCl dissolved in ddH_2O adjusted to pH 8.4. The following day plates were washed with PBST to remove any excess/unbound antigen and then incubated in a 200µL blocking solution containing 5%v/v FCS, PBST for 2 hr at 37°C. Sera and lavage samples were serially diluted in PBST, leaving a final volume of 50µL and incubated at 37°C for 1 hr. Following incubation, plates were washed thoroughly with PBST and then incubated with a secondary antibody to detect Ig subclasses. Rabbit anti-mouse IgA and IgG antibodies (Southern Biotech), directly horseradish peroxidise (HRP)-linked, were diluted 1:1000 with blocking buffer and added to each well and incubated for 1 hr at 37°C. Rabbit anti-mouse IgG1 and IgG2a detection antibodies (Southern Biotech – In vitro Technologies Pty Ltd, Noble Park, Australia) were biotin-linked and required an additional streptavidin-HRP step for detection (Southern Biotech). Streptavidin-HRP, diluted 1:1000 in blocking buffer, was added to each well and incubated at 37°C for 1 hr. 50µL of 3,3,5,5-tetramethylbenzidine (TMB) (HRP substrate) in a phosphate citrate buffer (PCB) (Sigma-Aldrich) was added to each well for 10 min. The colorimetric reaction between HRP and TMB was stopped by the addition of 50µL of 1M HCl. The OD was measured at 450nm on a xMark® microplate spectrophotometer (Biorad) and

endpoint titres were calculated for all samples using background absorbance of PBST plus two SD. Only groups receiving a MOMP-based vaccine induced antigen-specific antibodies, whereas those in unimmunised and adjuvant only control groups did not (data not shown).

In vitro neutralisation of *C. muridarum* infectivity

Lavage and serum samples, diluted 1/10, were each incubated with 10^3 IFU of purified *C. muridarum* in a 100µl volume for 1 hr at 37°C with 5% CO_2. This suspension was then applied to a confluent McCoy monolayer and incubated for 3 hr. Following incubation the antibody/*Chlamydia* suspension was removed, the monolayer washed with PBS and replaced with media containing 1µg/ml of cycloheximide. Infection was stopped and stained as described in Chapter 3. Percentage neutralisation was determined using the equation % neutralisation = [% cells infected (immune sample) - % cells infected (pre-sample)] / % cells infected (no sample).

RESULTS

Toxicity

Adjuvant safety is an essential consideration for human vaccines. We therefore determined the percentage of animals surviving each immunisation strategy by considering the number of animals requiring euthanasia due to vaccine-related sickness. Animals immunised with CTA1-DD, given with or without the MOMP, had a 100% survival rate regardless of route of administration (Figure 4.2). Conversely, IN delivery of CT/CpG result in only a 78% and 95% survival rate when given with or without the antigen, respectively. However, no animals immunised with the CT/CpG-based vaccine by both SL and TC required euthanasia due to sickness.

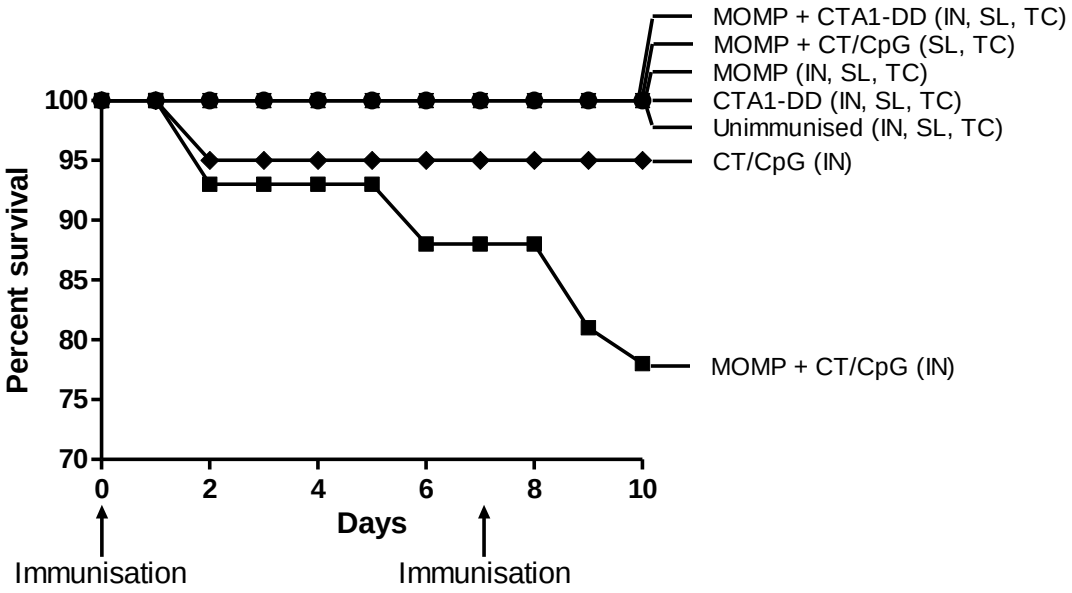


Figure 4.2: Percentage survival of mice following immunisation.
The percentage survival refers to the percentage of mice remaining from a specific group, as some mice required euthanasia due to a deterioration of their health following immunisation. The overall condition of each animal was assessed daily following vaccination. Animals displaying multiple signs of distress were euthanised immediately. No animals needed to be euthanised beyond day 10 after the initial immunisation.

Antigen-specific proliferation and cytokine production following *in vitro* stimulation with the MOMP

Antigen-experienced cells proliferate rapidly in response to an antigenic stimulus, due to decreased co-stimulatory requirements (Seder and Ahmed, 2003). We therefore examined antigen-specific proliferation, following *in vitro* re-stimulation with recombinant MOMP, to assess whether vaccination induced a MOMP-specific recall response. Cytokines produced during expansion following MOMP stimulation indicates how antigen-experienced cells will react when the MOMP is detected *in vivo*, following an infection. Specific cytokines can also determine the polarisation of the immune response (pro- or anti-inflammatory) and identify the cell types proliferating following stimulation, eg. T cells. As mentioned, protection against chlamydial infection is predominantly reliant on a pro-inflammatory cell-mediated response (Farris and Morrison, 2011), therefore generating T cells secreting cytokines like IFNγ is vital for an efficacious vaccine. We measured a number of cytokines predominantly produced by T cells (IFNγ, IL-4 and IL-17), in addition to other inducers and regulators of inflammation (TNFα and IL-10, respectively). Furthermore, proliferation and cytokine production by lymphocytes isolated from the spleen or the lymph nodes draining the genital and respiratory tracts were used to indicate the induction of a systemic or mucosal response, respectively.

Spleen

Intranasal immunisation with either vaccine (CTA1-DD or CT/CpG-containing) elicited a significant level of MOMP-specific proliferation by splenocytes when compared to the unimmunised control group ($P<0.001$) (Figure 4.3). Immunisation with the CT/CpG-based vaccine by the IN route induced a significantly greater amount of proliferation than the CTA1-DD-based vaccine, delivered via the same route ($P<0.001$). Antigen-specific proliferation was also significantly elevated in animals immunised with the CT/CpG-based vaccine by the SL ($P<0.01$) and TC routes ($P<0.001$) when compared to the unimmunised, but not when immunised with the CTA1-DD adjuvanted vaccine by the same routes. Levels of antigen-specific proliferation were highest following IN vaccination, indicating a greater potential of this route to induce a systemic response. Immunisation with the CT/CpG adjuvanted

vaccine consistently elicited a greater antigen-specific systemic recall response than CTA1-DD in the spleen, regardless of the route of immunisation.

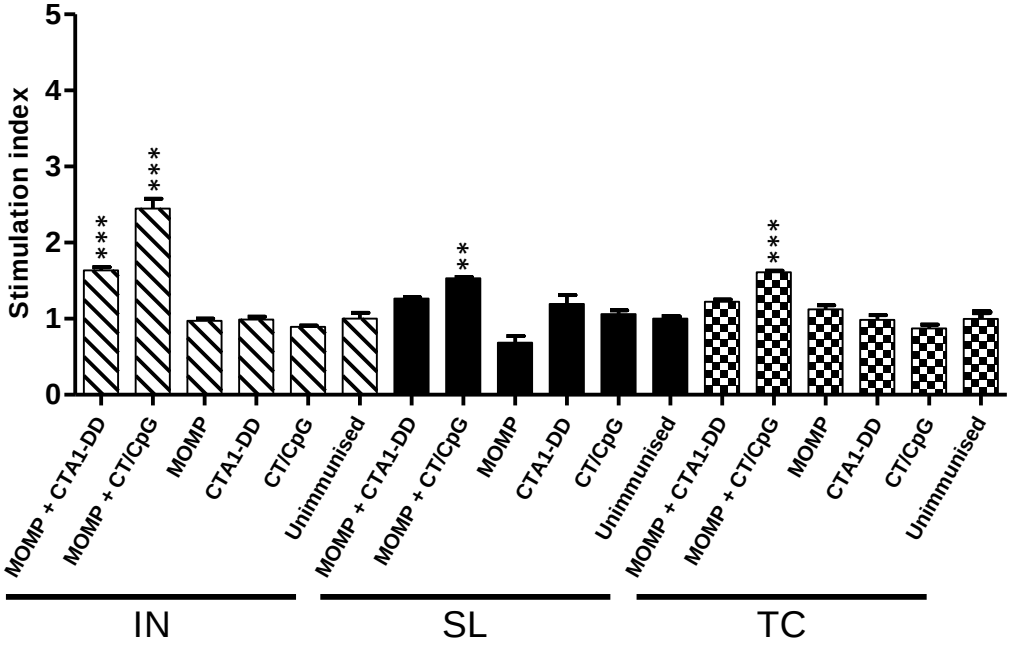


Figure 4.3: Antigen-specific proliferation by cells isolated from the spleen and stimulated *in vitro* with the MOMP.

The spleens were excised from vaccinated animals, pooled and homogenised to create a single-cell suspension. Splenocytes were stimulated with the MOMP or media for 72 hr and then incubated with thymidine for an additional 14 hr. Cell proliferation was determined by incorporation of thymidine into newly synthesised DNA. The cpm value (ionising radiation) of the MOMP stimulated cells was divided by the cpm value obtained from the media stimulated cells to give a stimulation index. The stimulation indexes for each vaccine are grouped with their respective routes of immunisation, IN (▨), SL (■) and TC (▩). Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

Splenic lymphocytes isolated from animals immunised via the IN route secreted the greatest quantity of cytokines following stimulation (Figure 4.4), which reflects the trends seen in proliferation (Figure 4.3). Immunisation with CT/CpG adjuvanted vaccines, by any route, resulted in significant cytokine secretions following *in vitro* stimulation with recombinant MOMP. The CTA1-DD adjuvanted vaccine effectively induced a cytokine response by splenocytes, when delivered via the IN and TC routes. Production of IFNγ was equal in animals receiving either IN delivered

vaccines, but this was significantly less in mice immunised topically with the CT/CpG-based vaccine ($P<0.05$). The cytokine IFNγ was not produced by stimulated splenocytes isolated from animals immunised with the CTA1-DD adjuvanted vaccine, delivered by routes other than IN. Significant levels of TNFα were also detected in all groups immunised with the CT/CpG-based vaccines regardless of route ($P<0.001$). Immunisation with the CTA1-DD-containing vaccine primed splenocytes to secrete a significant level of TNFα following re-stimulation, but only when the vaccine was delivered intranasally ($P<0.001$). Antigen-specific IL-17 production was strongest in IN vaccinated animals and was equivalent between vaccines. With the exception of the SL delivered CTA1-DD containing vaccine, IL-17 was also secreted by splenocytes isolated from all other vaccine groups, but at lower levels than IN immunised groups. Immunisation with the CTA1-DD adjuvanted vaccine via the TC route only induced a significant amount of IL-17 ($P<0.001$). With the exception of the IN administered CT/CpG adjuvanted vaccines, no vaccine induced a significant amount of IL-10. The IL-4 cytokine was not detected in any vaccine group at levels above unimmunised controls. This indicates that antigen-specific splenocytes isolated from mice immunised with either vaccine showed a dominant pro-inflammatory phenotype (IFNγ, TNFα and IL-17), as opposed to an anti-inflammatory response (IL-4 and IL-10), following *in vitro* stimulation with the MOMP.

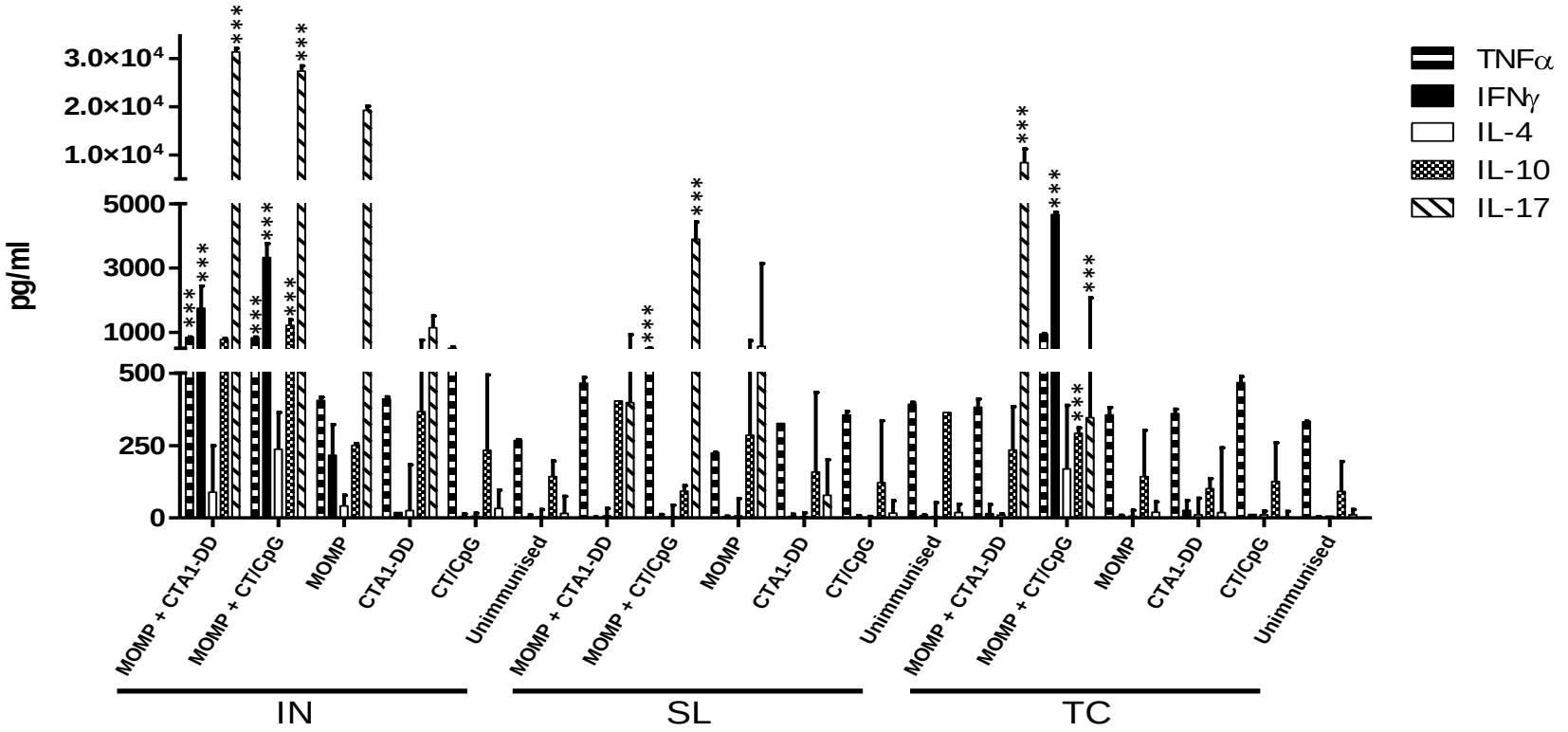


Figure 4.4: Antigen-specific cytokine production by cells isolated from the spleen and stimulated *in vitro* with the MOMP.

The amount of TNFα, IFNγ, IL-4, IL-10 and IL-17 (pg/ml) produced by splenocytes following stimulation with the MOMP was quantified from the media supplementing the proliferating cells using Bioplex® and ELISA. Both vaccines are grouped with their respective routes of immunisation, IN, SL and TC. Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

MdLN – draining the respiratory tract

Immunisation with the CT/CpG-based vaccine by the SL (P<0.01) and TC (P<0.001) routes, but not via the IN route, significantly increased antigen-specific proliferation by cells isolated from the MdLN following *in vitro* stimulation (Figure 4.5). The CTA1-DD-based vaccine administered topically generated a proliferative response in the MdLN significantly higher than control animals (P<0.05), but not when the vaccine was delivered via the IN or SL routes. CT/CpG delivered by SL (P<0.05) and TC (P<0.01) routes was however, significantly more effective than CTA1-DD at generating MOMP-specific recall in the MdLN. Levels of antigen-specific proliferation in the MdLN were greatest in animals receiving vaccines via SL and TC routes, highlighting the ability of these routes to prime and localise immunity in the lymph nodes draining the lungs.

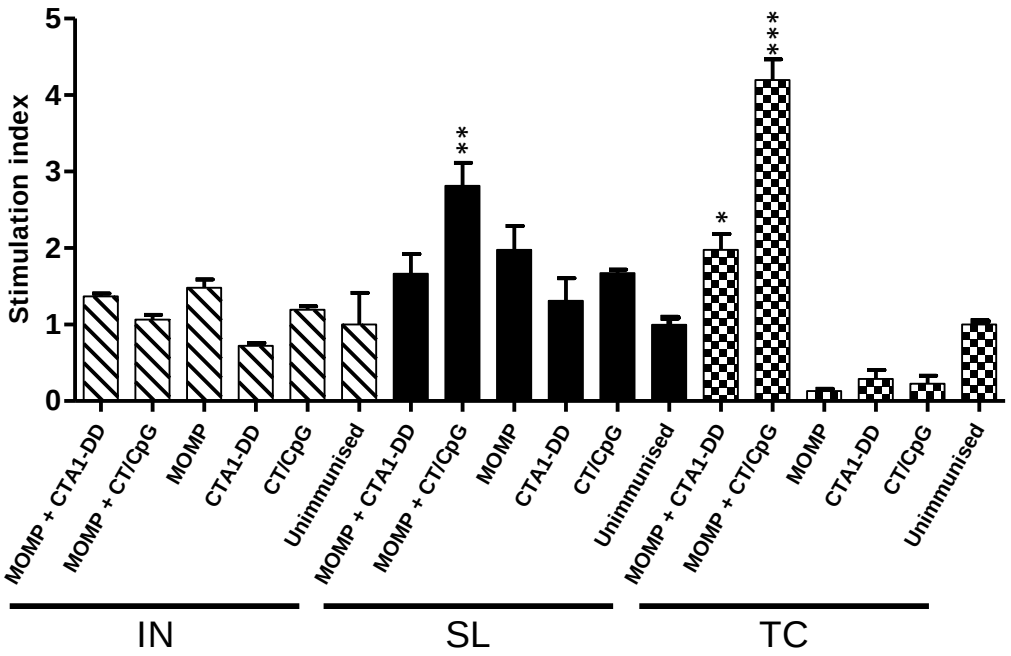


Figure 4.5: Antigen-specific proliferation by cells isolated from the MdLN and stimulated *in vitro* with the MOMP.

The MdLN were excised from vaccinated animals, pooled and homogenised to create a single-cell suspension. Cells were stimulated with the MOMP or media for 72 hr and then incubated with thymidine for an additional 14 hr. Cell proliferation was determined by incorporation of thymidine into newly synthesised DNA. The cpm value (ionising radiation) of the MOMP stimulated cells was divided by the cpm value obtained from the media stimulated cells to give a stimulation index. The stimulation index for each vaccine are grouped with their respective routes of immunisation, IN (▨), SL (■) and

TC (▪). Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

The vaccines that generated antigen-specific splenocytes, capable of proliferating and secreting cytokines upon stimulation, were quite different from those seen in the MdLN (Figure 4.6). Neither vaccine immunised intranasally elicited a significant cytokine response in the MdLN. Elevated cytokine levels were only detected in those groups previously shown to have significantly increased proliferation in the MdLN in response to the MOMP stimulation (Figure 4.5). This included both groups of animals immunised by the TC route and the group immunised with the CT/CpG-based vaccine via the SL route. Lymphocytes isolated from these groups predominantly secreted IL-17 following stimulation. The cytokine IFNγ was only detected in groups immunised via the TC route, although significance was limited to the CT/CpG-based vaccine. Cytokines TNFα, IL-4 and IL-10 were not found to be significantly elevated in any group. This indicates that of the vaccines that generated a MOMP-specific response in the MdLN, all lymphocytes produced a dominant pro-inflammatory response upon re-stimulation.

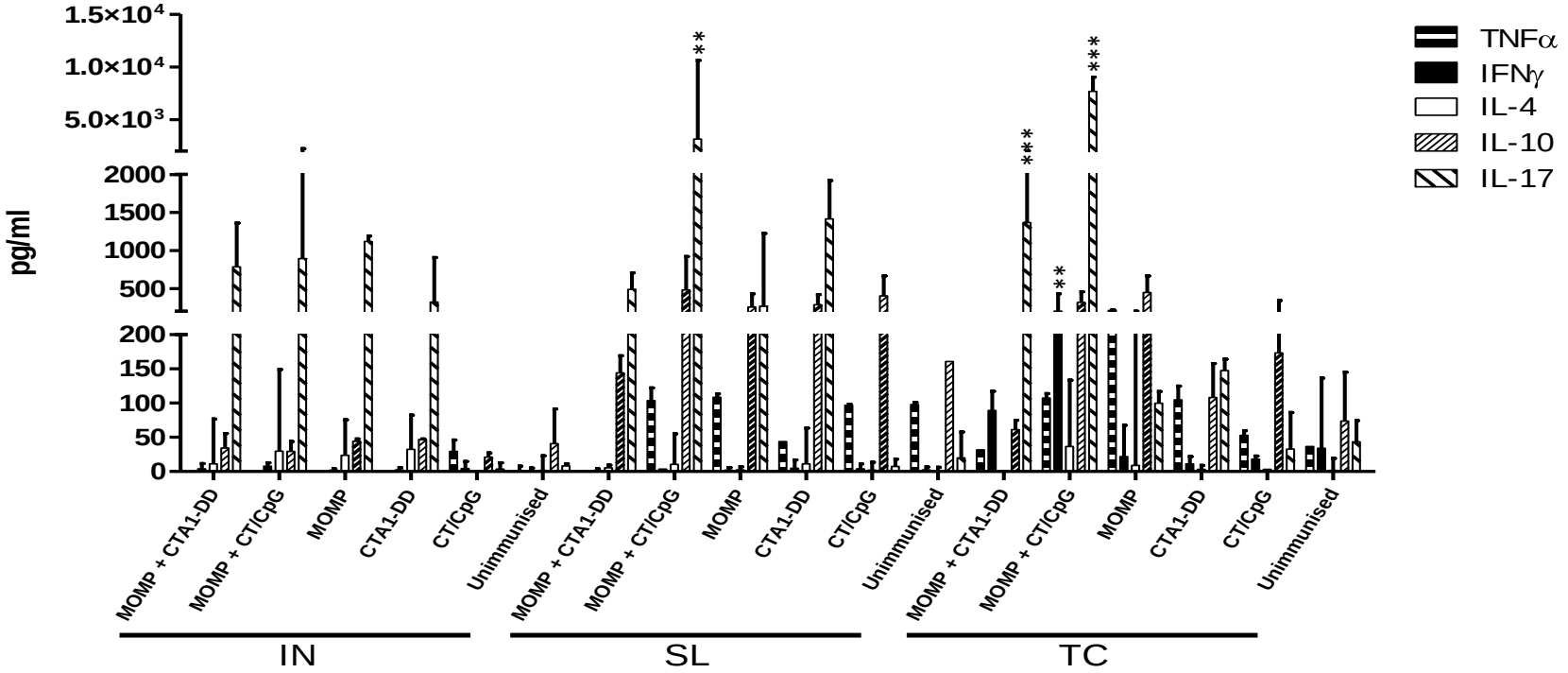


Figure 4.6: Antigen-specific cytokine production by cells isolated from the MdLN and stimulated *in vitro* with the MOMP.

The amount of TNFα, IFNγ, IL-4, IL-10 and IL-17 (pg/ml) produced by lymphocytes isolated from the MdLN following stimulation with the MOMP was quantified from the media supplementing the proliferating cells using Bioplex® and ELISA. Both vaccines are grouped with their respective routes of immunisation, IN, SL and TC. Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

MiLN – draining the reproductive tract

Immunisation with the CT/CpG-based vaccine by the TC (P<0.001) and IN (P<0.01) routes, but not via the SL route, significantly increased antigen-specific proliferation by cells isolated from the MiLN following *in vitro* stimulation (Figure 4.7). The CTA1-DD-based vaccine administered by the SL (P<0.001) and TC (P<0.001) routes also generated a proliferative response in the MiLN significantly higher than controls, but not when the vaccine was delivered via the IN route. The greater potential of CT/CpG to generate antigen-specific proliferation compared to CTA1-DD, seen previously in the spleen and MiLN, was not as obvious in the MiLN. The MOMP plus CTA1-DD was significantly more effective than the MOMP with CT/CpG when delivered by the SL route at generating MOMP-specific recall in the MiLN (P<0.05), although this difference was reversed following IN immunisation (P<0.01). Levels of antigen-specific lymphocyte proliferation in the MdLN were greatest in animals receiving vaccines via SL and TC routes, highlighting the ability of these routes to prime and localise immunity near the genital tract.

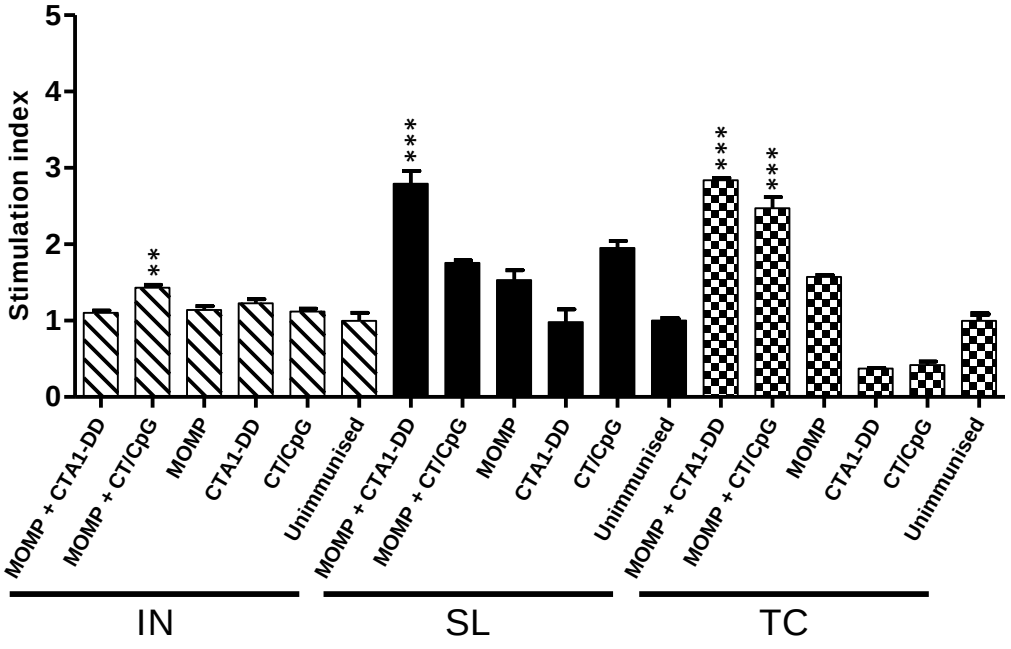


Figure 4.7: Antigen-specific proliferation by cells isolated from the MiLN and stimulated *in vitro* with the MOMP.

The MiLN were excised from vaccinated animals, pooled and homogenised to create a single-cell suspension. Cells were stimulated with the MOMP or media for 72 hr and then incubated with thymidine for an additional 14 hr. Cell proliferation was determined

by incorporation of thymidine into newly synthesised DNA. The cpm value (ionising radiation) of the MOMP stimulated cells was divided by the cpm value obtained from the media stimulated cells to give a stimulation index. The stimulation indexes for each vaccine are grouped with their respective routes of immunisation, IN (▨), SL (■) and TC (▣). Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

The vaccines that induced antigen-specific proliferation and cytokine production by lymphocytes isolated from the MiLN, differed from the vaccines that induced these same responses in both the spleen and MdLN. Cytokines were significantly elevated in groups immunised exclusively with the CT/CpG-based vaccine (Figure 4.8). Animals immunised with the CTA1-DD-based vaccine failed to elicit a cytokine response in the MiLN, regardless of route. All CT/CpG adjuvanted vaccines induced significant levels of TNFα in response to the MOMP stimulation ($P<0.001$), but only SL and TC delivered vaccines elicited significant levels of IFNγ ($P<0.001$). Production of IL-17 was again the dominant cytokine detected in all groups, yet only the CT/CpG-based vaccine delivered via the SL route elicited IL-17 at significant levels ($P<0.01$). High background levels of IL-17 were also detected in all animals in the TC group in the MiLN, which also drains the site of immunisation. No vaccine elicited a significant induction of IL-10 or IL-4. Consistent with the spleen and MdLN, lymphocytes isolated from the MiLN of vaccinated animals preferentially elicited a pro-inflammatory response, dominated by IFNγ, TNFα and IL-17.

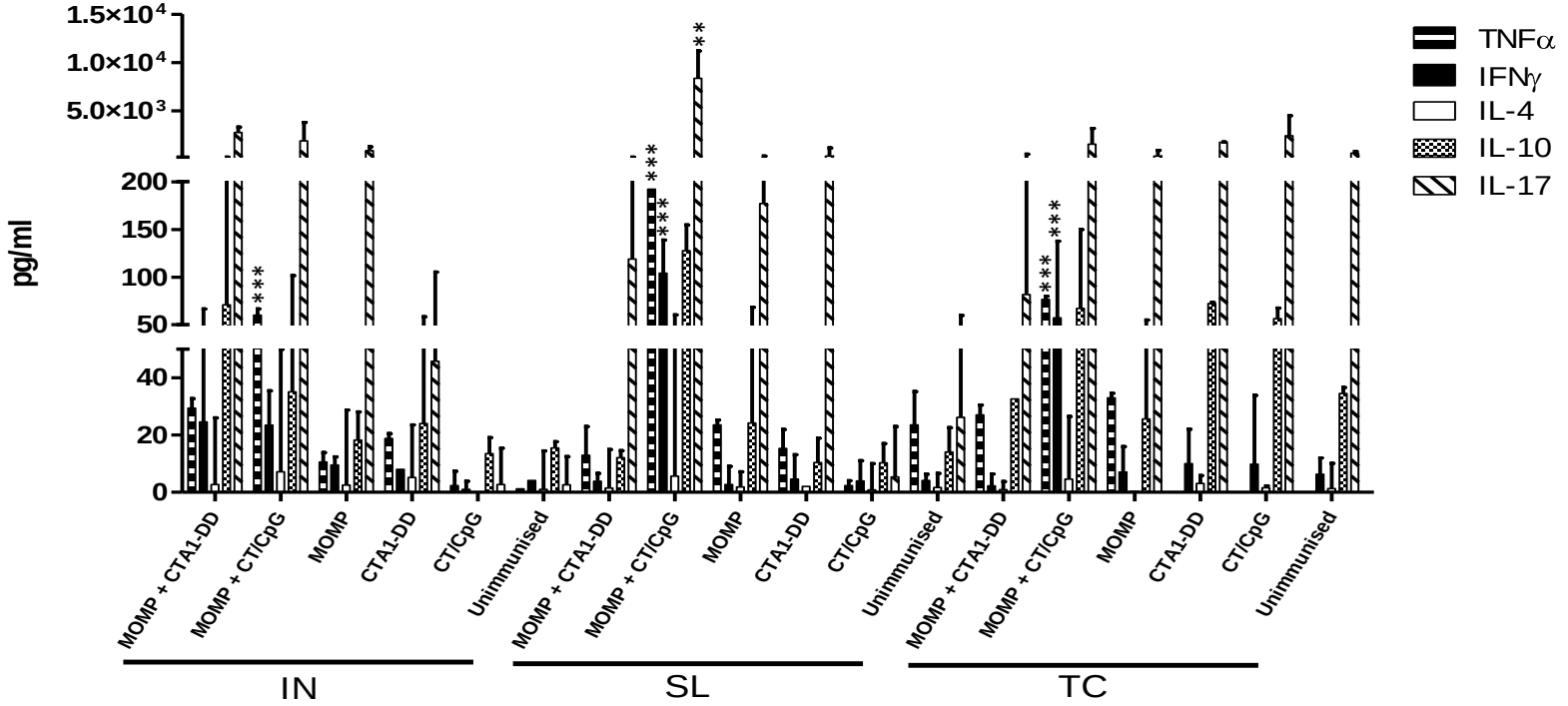


Figure 4.8: Antigen-specific cytokine production by cells isolated from the MiLN and stimulated in vitro with the MOMP.

The amount of TNFα, IFNγ, IL-4, IL-10 and IL-17 (pg/ml) produced by lymphocytes isolated from the MdLN following stimulation with the MOMP was quantified from the media supplementing the proliferating cells using Bioplex® and ELISA. Both vaccines are grouped with their respective routes of immunisation, IN, SL and TC. Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

Antigen-specific antibodies and *Chlamydia*-neutralising potential

The presence of *Chlamydia*-specific antibodies can also offer protection against the establishment of infection and pathology (Murthy *et al.*, 2004; Farris and Morrison, 2011). Therefore, vaccination should also aim to elicit a Th2-driven antibody response in addition to Th1-driven cell-mediated immunity. We quantified the levels of MOMP-specific systemic and mucosal antibodies following vaccination. Preventing a *Chlamydia* infection by inhibiting attachment to the host cell is the primary reason for generating MOMP-specific antibodies. Therefore, we also assessed the ability of serum and mucosal secretions to neutralise a chlamydial infection *in vitro*.

Serum – systemic antibodies

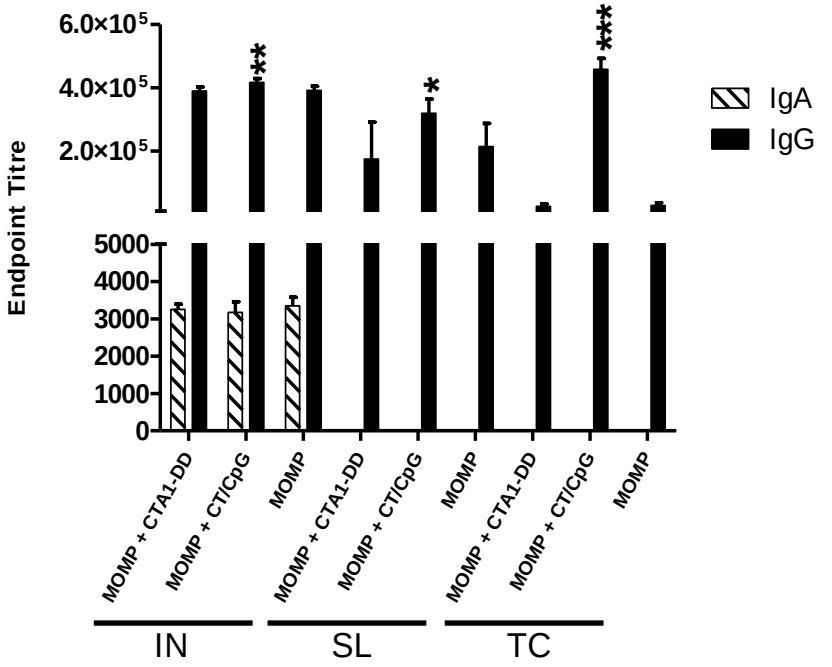
Serum IgG levels varied based on both adjuvant and immunisation route, but serum IgA production was unique to the IN route and independent of adjuvant (Figure 4.9). The CT/CpG containing vaccine elicited significantly greater MOMP-specific IgG levels in serum than CTA1-DD via all routes (P<0.05–0.001). Consistent with results seen previously (Cunningham *et al.*, 2009), the addition of the CTA1-DD adjuvant to a MOMP-based vaccine did not boost antigen-specific antibodies when compared to the antigen alone control. Overall, the IN route appeared superior to SL and TC routes at inducing a systemic antibody response. Interestingly, there was no significant difference in IgG titres between CT/CpG-based vaccines delivered via the IN or TC route.

The combination of adjuvants CT and CpG induced the desired balanced Th1/Th2 response via all routes as shown by the IgG2a:IgG1 ratios of 1:1 (Figure 4.9). Similarly, CTA1-DD also elicited a balanced Th1/Th2 response via the IN and SL route (1:1), but a more Th2-dominant response by the TC (1:5) route. Immunisation with the antigen alone in the absence of a polarising adjuvant by the IN (1:2) and TC routes (1:50) elicit a Th2-dominant response, whereas the SL route a more balanced response (1:1).

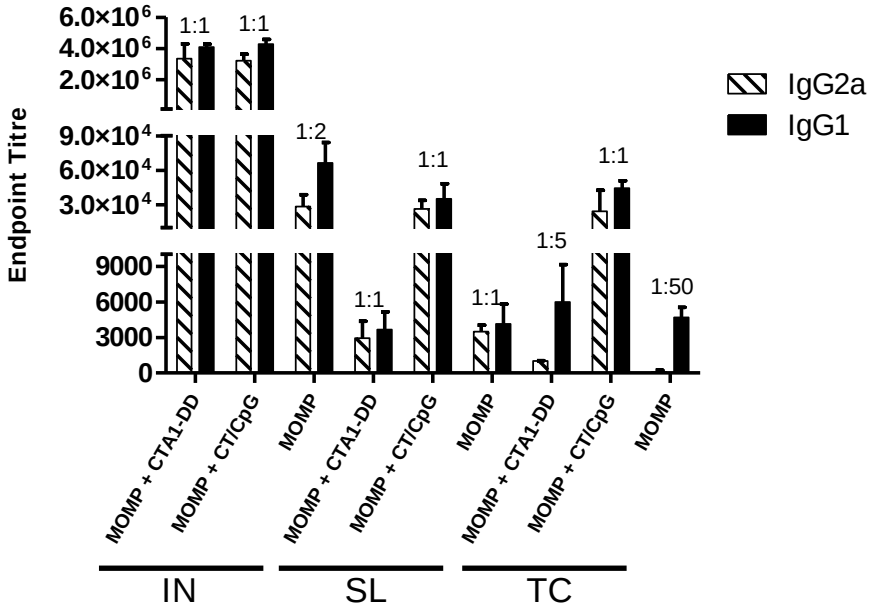
Although there were no significant differences between each vaccine delivered by the same route, both CTA1-DD and CT/CpG increased (between 10 – 50%) the

Chlamydia-neutralising capabilities of the serum when compared to the antigen only control (Figure 4.9). Despite the similarities in MOMP-specific antibody titres between the CTA1-DD-based vaccine and the antigen alone control, there was a significant increase in the neutralising capabilities in the serum of animals immunised with the CTA1-DD-containing vaccine (P<0.01–0.001). Moreover, there was a significant improvement in the neutralising potential of serum from animals immunised with the CT/CpG adjuvanted vaccine via the SL compared to TC route (P<0.01), despite the later group inducing significantly higher MOMP-specific IgG titres (P<0.01). The IN route was again the most effective when compared to SL and TC routes, for the induction of neutralising antibodies.

A.


Endpoint Titre
6.0×10⁵
4.0×10⁵
2.0×10⁵
5000
4000
3000
2000
1000
0
IgA
IgG
MOMP + CTA1-DD
MOMP + CT/CpG
MOMP
IN
SL
TC

B.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2.0×10⁶
9.0×10⁴
6.0×10⁴
3.0×10⁴
9000
6000
3000
0
1:1
1:2
1:5
1:50
IgG2a
IgG1
MOMP + CTA1-DD
MOMP + CT/CpG
MOMP
IN
SL
TC

C.

	MOMP + CTA1-DD (%)	MOMP + CT/CpG (%)	MOMP (%)
IN	80.48 ± 3.04 **	85.26 ± 2.46 ***	60.44 ± 1.75
SL	69.08 ± 4.16 ***	67.43 ± 7.51 ***	19.68 ± 8.14
TC	42.54 ± 2.37 **	40.26 ± 3.49 *	27.27 ± 3.86

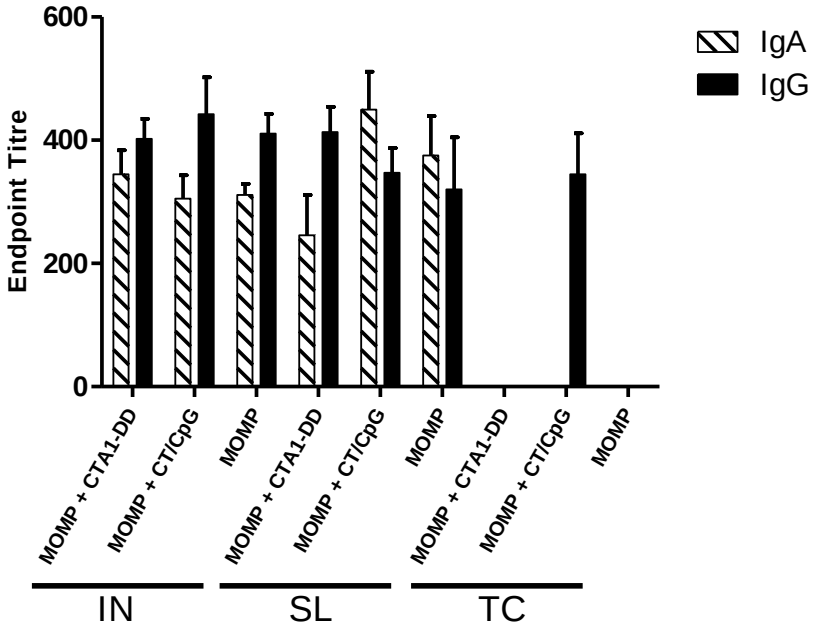
Figure 4.9: Antigen-specific systemic antibodies – serum.

The induction of MOMP-specific serum (A) IgG, IgA, (B) IgG1 and IgG2a following vaccination was quantified by ELISA. Endpoint titres were calculated for all samples using background absorbance of PBST plus two SD. (B) The ratio of IgG2a:IgG1, used to determine Th1:Th2 polarisation, is indicated above the titre bars in each group. (C) Percentage of infection neutralised *in vitro* was determined by incubation of *Chlamydia* with a 1/10 dilution of whole serum before quantification. Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

Bronchoalveolar lavage – mucosal antibodies in the respiratory tract

In the respiratory tract secretions there were no significant differences in antibody titres (IgG or IgA) between CT/CpG- and CTA1-DD-based vaccinated animals, with the exception of animals immunised via the TC route (Figure 4.10). No IgA was detected following TC immunisation; however, the CT/CpG-based vaccine did elicit a mucosal IgG response by the same route. IgA and IgG production was detected in the mucosal secretions of animals immunised via the IN and SL route. The neutralising capacity of BAL was enhanced by both adjuvants compared to immunisation with the MOMP alone, however only in the BAL from animals immunised by the SL route was this significant (P<0.05–0.01).

A.



B.

	MOMP + CTA1-DD (%)	MOMP + CT/CpG (%)	MOMP (%)
IN	15.22 ± 10.10	7.05 ± 7.09	2.21 ± 9.34
SL	29.31 ± 6.09 **	25.49 ± 6.66 *	1.66 ± 6.63
TC	5.44 ± 5.16	19.08 ± 10.73	4.70 ± 6.33

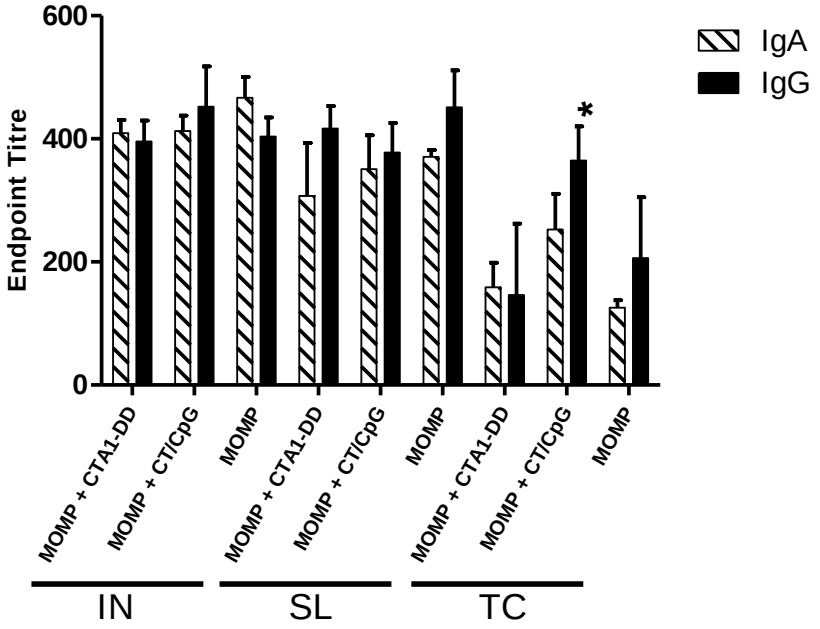
Figure 4.10: Antigen-specific mucosal antibodies – BAL.

(A) The induction of MOMP-specific IgG and IgA in the BAL following vaccination was quantified by ELISA. Endpoint titres were calculated for all samples using background absorbance of PBST plus two SD. (B) Percentage of infection neutralised *in vitro* was determined by incubation of *Chlamydia* with a 1/10 dilution of BAL before quantification. Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

Vaginal and uterine horn lavages – mucosal antibodies in the reproductive tract

With the exception of IgG in the TC immunised groups ($P<0.05$), there were no significant differences in antibody titres between vaccines detected in the lower reproductive tract (VL) (Figure 4.11). There were also no significant differences in IgA or IgG secretions between both vaccines by any route in the upper reproductive tract (UHL) (Figure 4.12). All animals immunised via the IN route consistently demonstrated MOMP-specific mucosal antibodies, whereas some animals in the SL and TC groups failed to respond, as indicated by the larger error bars. The TC immunised antigen only control group, also failed to elicit either antibody class in the uterine horn lavage. The MOMP-specific antibodies induced in the vaginal and uterine horn secretion following vaccination were not found to be significantly neutralising when compared to the antigen controls.

A.



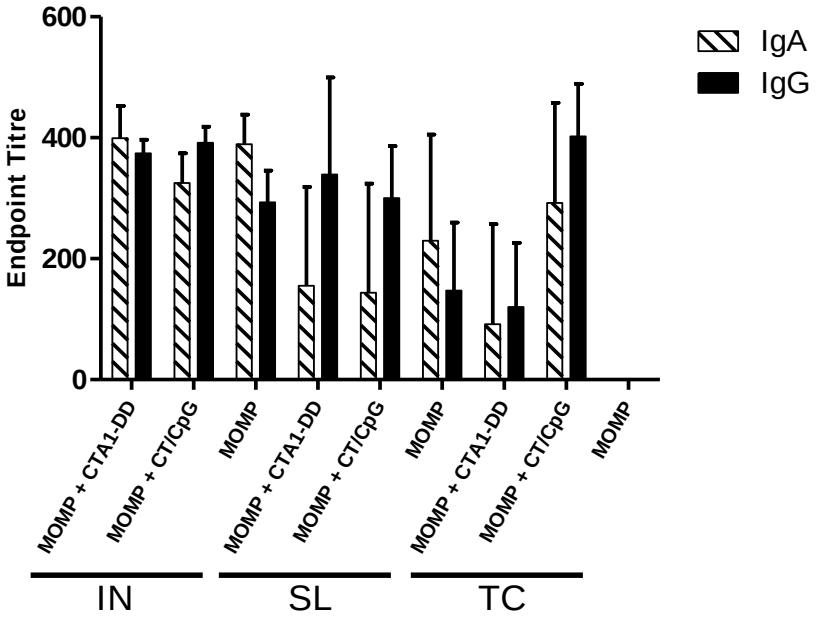
B.

	MOMP + CTA1-DD (%)	MOMP + CT/CpG (%)	MOMP (%)
IN	26.93 ± 46.04	11.84 ± 26.97	-1.903 ± 63.67
SL	23.45 ± 55.34	33.04 ± 54.55	-19.70 ± 34.80
TC	28.69 ± 52.97	28.24 ± 51.07	24.67 ± 15.30

Figure 4.11: Antigen-specific mucosal antibodies – VL.

(A) The induction of MOMP-specific IgG and IgA in the VL following vaccination was quantified by ELISA. Endpoint titres were calculated for all samples using background absorbance of PBST plus two SD. (B) Percentage of infection neutralised *in vitro* (± SD) was determined by incubation of *Chlamydia* with a 1/10 dilution of VL before quantification. Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

A.



B.

	MOMP + CTA1-DD (%)	MOMP + CT/CpG (%)	MOMP (%)
IN	16.01 ± 23.30	29.57 ± 17.17	39.04 ± 10.53
SL	10.89 ± 34.42	22.24 ± 15.28	19.73 ± 41.94
TC	13.82 ± 26.55	-46.10 ± 80.25	-24.17 ± 57.28

Figure 4.12: Antigen-specific mucosal antibodies – UHL.

(A) The induction of MOMP-specific IgG and IgA in the UHL following vaccination was quantified by ELISA. Endpoint titres were calculated for all samples using background absorbance of PBST plus two SD. (B) Percentage of infection neutralised *in vitro* (± SD) was determined by incubation of *Chlamydia* with a 1/10 dilution of UHL before quantification. Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

DISCUSSION

We immunised mice with the MOMP via three separate needle-free routes (IN, SL and TC) using two different adjuvants (CTA1-DD and CT/CpG) and compared antigen-specific cellular and humoral responses in both systemic and mucosal compartments. The CTA1-DD adjuvant delivered with or without the MOMP was found to be non-toxic in mice by all routes of immunisation. Conversely, CT/CpG administered alone or in conjunction with the MOMP was somewhat toxic by the IN route, but not via alternate routes (SL and TC). Following immunisation with both CTA1-DD- and CT/CpG-based vaccines, we could detect *Chlamydia*-neutralising antibodies in serum and mucosal secretions and MOMP-specific pro-inflammatory cells in the spleen and lymph nodes draining potential sites of infection. This is the desired balance between cell-mediated and humoral immunity identified in animal models to confer the greatest level of protection against the establishment of infection, transmission and pathology (Farris and Morrison, 2011). The route of immunisation affected the immunogenicity of each adjuvant, distribution of antigen-specific cells throughout the different lymphoid tissues, antibody class switching and the induction of mucosal antibodies. The adjuvant predominantly affected the polarisation (Th1, Th2 or Th17) of the immune response and the neutralising potential of antigen-specific antibodies.

The CT/CpG adjuvant was found to be toxic when delivered intranasally, an effect greatly exaggerated when administered together with the recombinant MOMP (Figure 4.2). The LPS contamination in the antigen preparation was reduced to a level below that is believed to be acceptable for mucosal immunisation (Eriksson *et al.*, 2004). However, CpG has been reported to enhance the development of toxic shock syndrome even with subpathogenic doses of LPS (Sparwasser *et al.*, 1997), which may have contributed to toxicity. The pathology reports (not shown) identified a significant influx of neutrophils and loss of epithelium integrity in the lungs and upper airways. This may suggest that trace amounts of the vaccine may have entered and caused inflammation in the lungs. Therefore, a probable cause of sickness in the mice that required euthanasia may have been a combination of toxic shock syndrome and pulmonary oedema, although this requires further investigation.

The route of immunisation influenced the effectiveness of each adjuvant; particularly CTA1-DD. Intranasal immunisation with each vaccine elicited a similar MOMP-specific immune response. However, immunisation with the CT/CpG-based vaccine consistently elicited stronger antigen-specific T and B cell responses than the CTA1-DD-containing vaccine, when both vaccines were administered by the SL or TC routes. This difference is likely linked closely with each adjuvant's mechanism of action, the APCs targeted and their density/distribution at the site of immunisation. The TLR9 molecule is expressed by keratinocytes, LCs, pDCs, mDCs, macrophages/monocytes, mast cells and B cells in mice (Wagner, 2004), which elicit a Th1-polarised response upon activation with CpG (Roman *et al.*, 1997; Jakob *et al.*, 1998). Cholera toxin binds all nucleated mammalian cells via the GM_1-ganglioside receptor to enhance Th2 as well as Th1 responses (Sanchez and Holmgren, 2010). Together, both CpG and CT elicit the desired balance between Th1 cell-mediated and Th2-driven humoral immunity. The success of this adjuvant combination by all routes of administration can be attributed to its capacity to target and stimulate a number of innate cell populations localised at the site of immunisation, thus allowing for superior uptake/presentation of co-administered antigens and development of adaptive immunity.

The CTA1-DD adjuvant is a non-toxic derivative of CT that retains many of the immunomodulatory properties of the holotoxin, i.e. Th1/Th2 polarising response, but differs greatly in target cell binding. The CTA1-DD adjuvant is thought to predominantly, but not exclusively, target B cells as APCs (Lycke, 2005). More recently, CTA1-DD has been shown to bind FDC in B cell follicles in a CR2-dependent manner and enhance GC reactions and T cell-dependent responses by activation of the complement pathway (Mattsson *et al.*, 2011). The immunogenicity of CTA1-DD, similar to CT, is highly dependent on binding and internalisation of the enzymatically active ADP-ribosyltranferase subunit for optimal adjuvanticity (Agren *et al.*, 1999b). This may explain why CTA1-DD is immunogenic via the IN route, which targets the B cell and FDC rich NALT, but is less effective when administered via the TC and SL sites, routes that target tissues where these targets cells are not normally found in great numbers in a resting state (Bos, 2005; Mascarell *et al.*, 2009). The immunogenicity of CTA1-DD has been enhanced by others

following immunisation via routes other than IN by (1) pre-incubating CTA1-DD with IgG to form immune complexes capable of degranulating mast cells (Fang *et al.*, 2010) and through (2) the incorporation of CTA1-DD into the immune-stimulating complexes (ISCOMs), thereby enhancing uptake by resident DCs (Helgeby *et al.*, 2006). Our attempts to improve the immunogenicity of CTA1-DD by alternate routes (TC immunisation) using a B cell chemoattractant (CXCL13), failed to significantly improve antigen-specific responses from that of our standard GM-CSF pre-treatment (Appendix 2). Therefore, CTA1-DD delivered via the IN route induced a similar Th1/Th2 balanced response to the CT/CpG adjuvant combination, without the toxicity associated with nasal delivery (Mutsch *et al.*, 2004), but the immunogenicity of CTA1-DD appears reliant on delivery by the IN route, abundant in known target populations, B cells and FDCs.

There was however some evidence to suggest that CTA1-DD was immunogenic via the TC route. Transcutaneous immunisation with the CTA1-DD-based vaccine generated a significantly higher amount of the T cell-related cytokine IL-17 (Figure 4.4, 4.6) and MOMP-specific IgG2a antibodies (Figure 4.9) when compared to the antigen alone control. The same improvements in antigen-specific responses were not detected following SL immunisation with the same vaccine, which suggests that the method of TC immunisation in some way improved the immunogenicity of CTA1-DD. Physical differences exist between the buccal epithelium and the stratum corneum of the skin in regards to permeability (Sohi *et al.*, 2009), although this should have favoured the induction of antigen-specific response following SL immunisation as opposed to topical delivery, contrasting to the effects found in our study. The skin and buccal mucosa also share considerable similarities in the variety and distribution of immune cells (Bos, 2005; Mascarell *et al.*, 2009), therefore the site of immunisation is unlikely to account for the significant differences observed in effectiveness of CTA1-DD following immunisation by the SL and TC routes. The methods of TC immunisation differed from SL immunisation in three main ways. Trancutaneous immunisation included (1) an extended duration of contact to the vaccine, (2) the chemoattractant GM-CSF and (3) chemical permeation enhancers, all of which are known to influence the generation of antigen-specific responses (Hickey *et al.*, 2005; Naito *et al.*, 2007; Karande *et al.*, 2009). Animals immunised

via the TC route were exposed to the vaccine for a period of 24 hours, compared to only one hour for SL immunised mice. Antigen-specific responses can be significantly enhanced simply by prolonging the presence of the antigen on the skin, even in the absence of an adjuvant (Naito *et al.*, 2007). By extending the length of time the vaccine was in contact with the skin, this may have allowed more time for B cells to be recruited to the site of immunisation and interact with the adjuvant. The chemoattractant GM-CSF used prior to TC immunisation has also been shown to increase T and B cell responses, by as much as 16-fold (Hickey *et al.*, 2005). This chemokines also recruits APCs (DCs and macrophages) that could have improved immunogenicity directly by rapidly internalising CTA1-DD (Agren *et al.*, 1997) or indirectly through cytokine/chemokines secretions responsible for recruiting B cell targets (Hamilton and Anderson, 2004). The method of skin permeation used prior to TC but not SL immunisation, included a combination cationic surfactant, azone-like and fatty-acid esters, chosen primarily for their ability to disrupt the stratified epithelium, improve the critical passage of the vaccine into the epidermis (Karande *et al.*, 2009) and facilitate the development of antigen-specific responses following TC immunisation (Appendix 1). As the buccal mucosa is considerably more permeable than the skin (Sohi *et al.*, 2009), SL immunisation does not require a prior pre-treatment with permeation enhancers to elicit a robust antigen-specific response (Cuburu *et al.*, 2007; Cuburu *et al.*, 2009). The permeation enhancers used prior to TC immunisation however also increases the production of IL-1α (Karande *et al.*, 2009), a known stimulator of T cell differentiation particularly to Th2 cells as well as IL-17-secreting Th17 cells (Helmby and Grencis, 2004; Ben-Sasson *et al.*, 2011). Large amounts of IL-17 could be detected in the MiLN draining the TC site of immunisation even in unimmunised animals receiving the pre-treatment only (Figure 4.8), which provides evidence for the non-specific Th17 polarising effect of the permeation enhancer or the TC route. The IL-17 cytokine is known to play an important role in the recruitment of B cells (Wang *et al.*, 2011a; Zhang *et al.*, 2011), essential for the optimal effect of CTA1-DD (Helgeby *et al.*, 2006). In addition, IL-17 can also modulate DCs to polarise a Th1 response, responsible for promoting IgG2a production (Bai *et al.*, 2009). Therefore, a synergistic relationship between the chemoattractant, the permeation enhancers and the even duration of vaccine

exposure, may have improved the immunogenicity of CTA1-DD by the TC route through the recruitment and stimulation of B cells.

The route of immunisation also influenced the dissemination of antigen-specific responses to distant mucosal compartments, i.e. genital and respiratory tract, potentially via the CMIS. Despite other studies reporting that lymphocyte trafficking can be manipulated by specific vaccine constituents (Enioutina *et al.*, 2009), our data suggests that antigen-specific cell homing was adjuvant-independent. Immunisation via the IN route induced a strong splenic antigen-specific response (Figure 4.3, 4.4). The SL and TC routes of immunisation on the other hand generated a systemic antigen-specific response, in addition to a more regional response in the lymph nodes draining the respiratory and reproductive mucosa (Figure 4.5 – 4.8). As a pathogen that infects through mucosal epithelium will usually be processed and presented by APCs to lymphocytes in the regional lymph nodes before reaching systemic sites like the spleen (Zhao *et al.*, 2003), antigen-specific cells residing in the draining lymph nodes may be better positioned to react and elicit an immune response earlier during the course of the infection. Recruitment of lymphocytes into lymph nodes is mediated by the pattern of addressins and chemokines expressed by the high endothelial venules (HEV), which are the main site of lymphocyte entry from the blood. Lymphocytes require specific selectins, integrins and chemokines receptors complementary to the unique signature of ligands expressed by the HEV to initiate rolling, activation, attachment and eventual transmigration into a particular lymph node (von Andrian and Mempel, 2003). Generally, entry of lymphocytes into the lymph nodes is considered to be CCR7- and L-selectin (CD62L)-dependent. T cells isolated from CCR7-deficient mice and adoptively transferred of into WT animals accumulate in the spleen, but not the lymph nodes (Forster *et al.*, 1999). Similarly, $CD62L^{-/-}$ mice also have a significant reduction in the numbers of lymphocytes in present in the peripheral lymph nodes (Arbones *et al.*, 1994). Neutralisation of CD62L dramatically reduces the quantity of T cells in the MiLN draining the reproductive tract, but not their accumulation in the spleen or MdLN (Ciabattini *et al.*, 2011). The α- and β-chains of the integrins can also influence lymphocyte migration (Berlin *et al.*, 1995; Steeber *et al.*, 1998), mediating selectin-independent capture from blood. The proportion of T cells expressing integrin $\alpha_4\beta_7$ and $\alpha_E\beta_7$ is

also elevated in the MiLN compared to other secondary lymphoid tissues (Gupta *et al.*, 2005; Martinelli *et al.*, 2011), suggesting that these integrins are required to enter the lymph nodes draining the genital tract. The homing phenotypes of T cells isolated from the spleen and the MdLN draining the lungs are remarkably similar, both of which appear dependent on expression of $\alpha_4\beta_1$ (Ferguson and Engelhard, 2010). However, neutralisation of $\alpha_4\beta_7$ affects the entry of lymphocytes into the spleen but not the MdLN (Ciabattini *et al.*, 2011). Therefore the passage of lymphocytes into the MiLN ($CCR7^+$, $CD62L^+$, $\alpha_4\beta_7^+$, $\alpha_E\beta_7^+$), the MdLN ($CCR7^+$, $CD62L^-$, $\alpha_4\beta_7^-$, $\alpha_4\beta_1^+$) and the spleen ($CCR7^-$, $CD62L^-$, $\alpha_4\beta_7^+$, $\alpha_4\beta_1^+$) each require a unique pattern of homing receptors. The route of immunisation has a significant impact on the expression of the various adhesion molecules and chemokines receptors by antigen-specific lymphocytes (Quiding-Jarbrink *et al.*, 1995; Kantele *et al.*, 1997; Quiding-Jarbrink *et al.*, 1997; Song *et al.*, 2009) (discussed further below). These modifications not only influence the localisation of the activated cells in secondary lymphoid tissues, but also their ability to be recruited to mucosal surfaces at times of inflammation and infection to elicit protection (Kelly and Rank, 1997; Perry *et al.*, 1998; Feng *et al.*, 2000; Kelly *et al.*, 2000; Kelly *et al.*, 2001; Kelly *et al.*, 2009). Therefore, both the SL and TC routes of immunisation appear to prime and position antigen-specific cells in key lymph nodes where chlamydial antigens will likely be presented first during an active infection.

In addition to influencing the distribution of antigen-specific cells, the route of immunisation also effected antibody class switching and the induction of mucosal antibodies. All vaccines elicited MOMP-specific IgG in serum and the levels of IgG in the mucosal lavages (Figure 4.10 – 4.12) mirrored trends seen in the serum (Figure 4.9), signifying that transudation from the blood was the likely source of mucosal IgG (Lencer and Blumberg, 2005; Mestecky *et al.*, 2005). However, the quantity of IgG in both the genital and respiratory tract lavages was similar across all groups, despite significant differences in their serum IgG levels. Perfusion of IgG at the mucosal surface appears to be limited in mice and has been reported to be a fraction of what is transported in humans (Wu *et al.*, 2000; Mestecky *et al.*, 2004; Li *et al.*, 2011b).

All immunisation routes elicited a mucosal IgA response, however only the IN route was capable of inducing systemic IgA (Figure 4.9). The NALT, targeted during IN immunisation, is abundant in GCs located beneath specialised antigen-processing M cells (Zuercher *et al.*, 2002). In general, T cell-dependent IgA B cell differentiation occurs most effectively in GCs (Cerutti, 2008), which stimulate the expression of the DNA-editing enzyme activation-induced cytidine deaminase (AID) in B cells responsible for antibody gene diversification, i.e. affinity maturation (somatic hypermutaton (SHM)) and CSR. Both the skin and buccal mucosa lack the organised lymphoid tissues similar to the NALT, thus fail to generate systemic IgA effectively (Kaspsenberg and Bos, 1998; Mascarell *et al.*, 2009). The induction of serum IgA by SL and TC delivered vaccines has however been reported by others using similar adjuvants (Maeba *et al.*, 2005; Cuburu *et al.*, 2007; Cuburu *et al.*, 2009; Hervouet *et al.*, 2010). The antigen, specifically the size of the antigen, appears to be an important factor in these studies allowing the induction of systemic IgA. Ovalbumin, gp41 and the *Porphyromonas gingivalis* outer membrane protein together with CT all successfully generated serum IgA, albeit low levels, following SL or TC immunisation. These antigens range in size from 40 – 45kDa, while recombinant MOMP is almost double the size at 81kDa. Transcutaneous immunisation with bovine serum albumin (BSA) (67kDa) and recombinant MOMP have shown a similar lack of antigen-specific serum IgA, despite utilising epithelial cell permeabilising adjuvants (John *et al.*, 2002; Berry *et al.*, 2004; Skelding *et al.*, 2006; Rollenhagen *et al.*, 2009), Therefore, the inability of SL and TC applied vaccines to induce an antigen-specific serum IgA response, may reflect the capacity of the antigen to enter the epithelium, migrate to the draining lymph nodes and interact with GCs (Sabirov and Metzger, 2008).

The size of the antigen appeared to be a disadvantage for the induction of systemic IgA, although genetically linking the MOMP with MBP improves the solubility and correct conformational folding of the recombinant protein (Su *et al.*, 1996; Fox *et al.*, 2001), vital for production of neutralising antibodies (Sun *et al.*, 2009). Moreover, MBP also acts as an adjuvant (Kushwaha *et al.*, 2001; Simmons *et al.*, 2001a; Simmons *et al.*, 2001b; Fernandez *et al.*, 2007; Yuzawa *et al.*, 2012). Proteins fused with MBP generate significantly more serum antibodies than untagged proteins

(Simmons *et al.*, 2001a; Simmons *et al.*, 2001b; Yuzawa *et al.*, 2012), as MBP is a TLR4 agonist that stimulates cytokine production and expression of co-stimulatory molecules on DCs (Fernandez *et al.*, 2007). This provides an explanation for why needle-free immunisation with recombinant MOMP, in the absence of an adjuvant, stimulates a strong antigen-specific response as opposed to mucosal tolerance (Figure 4.9) (Czerkinsky *et al.*, 1999; Fujihashi and McGhee, 2004).

Mucosal antigen-specific IgA was detected in the lung secretions of SL immunised animals, in the absence of serum IgA (Figure 4.9, 4.10). Similarly, IgA was also detected in both upper and lower genital tract secretions following immunisation via SL and TC routes (Figure 4.11, 4.12), again despite the lack of any serum IgA. The majority of IgA detected in mucosal secretions is produced locally by plasma cells rather than by active transport from the serum (at least in the gastrointestinal tract) (Jonard *et al.*, 1984), which implicates a mucosal homing phenotype imprinted on B cells by each route of immunisation. The dissemination of antigen-specific responses initiated in the SL mucosa to distant mucosal compartments is hypothesised to be dependent on $CCR7^{+}CD11c^{+}$ DCs. This subset primes T and B cells to migrate towards the chemokine CCL28, expressed by epithelial cells at numerous sites in the body including the genital and respiratory tract mucosa (Song *et al.*, 2009; Czerkinsky and Holmgren, 2010a). Similarly, DCs process Vitamin D3 in the skin to its active form $1,25(OH)_2D_3$, which activates CCR10 expression and hence attraction of primed lymphocytes to the epidermal produced chemokine CCL27 (Sigmundsdottir *et al.*, 2007). The chemokines receptor CCR10 is promiscuous in nature and can also respond to CCL28 (Wang *et al.*, 2000; Homey *et al.*, 2002), the same chemokines driving migration of lymphocytes activated following SL immunisation. This may explain why antigen-specific responses are similar between animals immunised by SL and TC routes, due to the equal dependence on the CCR10 and CCL27/CCL28 pathway for migration. Intranasal vaccination induced IgA in all mucosal secretions tested. We cannot however definitively determine the source of the mucosal antibodies, i.e. whether IgA transfused from the serum into the lumen or if it was locally produced. Antigen-specific antibody-secreting cells can localise in genital tract and lung tissues following IN immunisation (Berry *et al.*, 2004; Skelding *et al.*, 2006). Increased expression of the mucosal homing integrin $\alpha_4\beta_7$ on

antibody-secreting cells has been documented following IN immunisation (Quiding-Jarbrink *et al.*, 1997), although homing to the genital at least appears to be β_7-independent (Goodsell *et al.*, 2008). Immunoglobulian A-secreting plasma cells generated following IN immunisation have also been found to express CCR10 and migrate towards CCL28 (Cha *et al.*, 2011). Therefore, all vaccines were able to induce the production of mucosal IgA, potentially by stimulating similar pathways of B cell recruitment to mucosal tissues.

The consistent mucosal IgA production by all animals generally favoured vaccines containing the CT/CpG adjuvant and those delivered by the IN route (Figure 4.10 – 4.12). B cell differentiation into IgA-secreting cells at the systemic level is largely believed to be dependent on the formation of GCs, whereas mucosal IgA production is governed by a different mechanism (Schubart *et al.*, 1996; Kosco-Vilbois *et al.*, 1997; Tarlinton, 1998; Gardby *et al.*, 2003). Mucosal IgA can be generated in the absence of NALT, GC formation and various other cytokines and co-stimulatory molecules required for producing systemic antibodies (Vajdy *et al.*, 1995; Neumann *et al.*, 1996; Gardby *et al.*, 2003; Sabirov and Metzger, 2008). Expression of AID, responsible for SMH and CSR, is absolutely essential for production of both serum and mucosal IgA (Wei *et al.*, 2011). Signals that induce AID-mediated CSR include BCR ligation, NF-κB induction signals (CD40 or TLRs), transcription factors and cytokines that promote B cell differentiation and/or B cell survival (Bemark *et al.*, 2012). Attenuation of mucosal and systemic antibodies has also been reported in CD80, CD86 and CD80/CD86 double-deficient mice (Garcia *et al.*, 2004), highlighting the importance of providing adequate co-stimulation to B cells for production of mucosal IgA. As mentioned above, CTA1-DD appeared less immunogenic following topical or SL delivery. Therefore, an ability activate of AID and mature B cells may offer an additional reason why the CTA1-DD containing vaccine delivered by routes other than IN were unable to consistently generate IgA in all animals and mucosal secretions.

In addition to the actual presence or absence of antibodies, as determined by ELISA, adjuvant choice and route of delivery also affected antibody function. We addressed this by comparing the capacity of serum and mucosal antibodies to neutralise a

chlamydial infection *in vitro* (Figure 4.9 – 4.12). The ability of an antibody to neutralise is thought to be dependent on the quantity, affinity and avidity of the antibodies (Steward *et al.*, 1991; Chargelegue *et al.*, 1995; Olszewska *et al.*, 2000). Antibody affinity is defined by the strength of a single antibody bond determined by a dissociation coefficient, which is enhanced during affinity maturation by the rate of SHM (Wabl *et al.*, 1999). Antibody avidity is the combined strength of multiple bonds and this is predominantly influenced by the isotype of the antibody and the number of binding sites the antibody possess (IgG = 2 and IgA = 4). Serum from IN vaccinated animals possessed a far superior *Chlamydia* neutralising capability than SL and TC immunised groups. The presence of IgA in the serum of IN immunised groups may account for this increase in neutralising capacity, through induction of more avid antibodies (Peterson *et al.*, 1993; Pal *et al.*, 1997b). Moreover, MOMP-specific IgA is thought to reduce infectivity by preventing chlamydial attachment to the host cell (Cunningham *et al.*, 2008). This function is unaffected by the restrictions of *in vitro* culture, as neutralisation only requires the presence of antibodies to prevent attachment. Alternatively, serum from SL and TC vaccinated animals, which contained only IgG, failed to neutralise *Chlamydia in vitro* as effectively as the serum from IN immunised mice. This is because MOMP-specific IgG-dependent protection against *Chlamydia* occurs through opsonisation and phagocytosis (absent from the *in vitro* neutralisation assay) and not via direct inhibition of attachment or complement activation (Caldwell and Perry, 1982; Moore *et al.*, 2002). Therefore, IgA is more capable of neutralising *Chlamydia,* at least *in vitro*.

Interestingly, in some instances there was no direct correlation between MOMP-specific antibody titres and neutralisation. The CT/CpG-based vaccine delivered by the TC routes generated a significantly greater systemic IgG response than the same vaccine delivered by the SL route (Figure 4.9). Serum from the SL vaccinated groups however, exhibited higher neutralisation of infection *in vitro*. The reason for this is unknown, but could reflect the more permeable nature of the buccal mucosa (Sohi *et al.*, 2009), facilitating better antigen uptake, APC maturation, SHM and resulted higher affinity antibodies.

Intranasal immunisation with the CTA1-DD-based vaccine elicited an equivalent serum MOMP-specific antibody response to the antigen alone group immunised by the same route as determined by ELISA, yet neutralisation favoured the CTA1-DD adjuvanted vaccine (Figure 4.9). This has been reported previously and was suggested to be the result of direct interaction between the adjuvant and B cells (Cunningham *et al.*, 2009). The CTA1-DD adjuvant is known to up-regulate expression of CD86 and CD80 on B cells responsible for generating GCs and long-term affinity matured memory B cells (Agren *et al.*, 1997; Agren *et al.*, 1999b; Bemark *et al.*, 2011). Therefore, the route of immunisation used influenced neutralisation, so too did the adjuvant.

CONCLUSIONS

The aim of this chapter was to characterise and compare the systemic and mucosal immune responses in the genital and respiratory tracts following immunisation with the MOMP plus CTA1-DD or CT/CpG via TC, IN and SL routes. The CT/CpG adjuvant can be used safely via routes other than IN to effectively elicit a robust cell-mediated and humoral immune response. The CTA1-DD adjuvant is safe by all routes, but most immunogenic following IN delivery. The immune responses induce following immunisation with either vaccine were comparable after IN administration, but the greater induction of antigen-specific responses favoured the CT/CpG adjuvanted vaccine via the alternate SL and TC routes. The route of immunisation predominantly determined the distribution of T and B cells to different effector sites, potentially via the common mucosal immune system, whereas the adjuvant influenced the quality and quantity of antigen-specific cellular and humoral responses. However, the efficacy of each vaccine can only be truly measured following a live challenge with *Chlamydia*.

CHAPTER FIVE: PROTECTION AGAINST A RESPIRATORY TRACT INFECTION AND THE ASSOCIATED PATHOLOGY FOLLOWING AN INTRANASAL CHALLENGE WITH *C. MURIDARUM*

INTRODUCTION

C. pneumoniae is a respiratory tract pathogen transmitted from person to person in water droplets propelled by coughing or sneezing. Serological evidence suggests that virtually everyone will be infected with *C. pneumoniae* at one point in their lifetime (Grayston, 2000). *C. pneumoniae* infections are primarily asymptomatic, but in 30% of cases, can develop into a severe respiratory tract illness like pneumonia, of which *C. pneumoniae* is the third most common cause (Kuo *et al.*, 1995; Grayston, 2000). Persistent infections can exacerbate chronic inflammatory diseases like asthma, COPD, CVD, Alzheimer's disease, MS and reactive arthritis (Kuo *et al.*, 1993; Clementsen *et al.*, 2002; Belland *et al.*, 2004; Bachmaier and Penninger, 2005; Blasi *et al.*, 2009). As 75% of first infections occur in early life, this highlights the need for early intervention through vaccination to prevent infection and exacerbation of chronic inflammatory diseases (Aldous *et al.*, 1992; Miyashita, 2006).

Development of an effective human vaccine relies on successful vaccine trials in animal models that replicate human infection. The *C. muridarum* mouse model of infection replicates many aspects of human infection and disease (Fan *et al.*, 1999; Horvat *et al.*, 2007; Rey-Ladino *et al.*, 2007; Jupelli *et al.*, 2008; Kaiko *et al.*, 2008; Horvat *et al.*, 2010). Mice can be infected with *C. muridarum* by inhalation, the natural route of infection in humans (Ramsey *et al.*, 2009). Infected animals undergo a stage of dramatic weight loss known as cachexia, which is an indicator of chronic inflammation and development of pneumonia (van Heeckeren *et al.*, 2000). *C. muridarum* can also induce intense thickening and fibrotic scarring of the airways associated with COPD, allergic sensitisation and exacerbation of asthma, by manipulating Th2-related cytokine production (Horvat *et al.*, 2007; Kaiko *et al.*, 2008; Horvat *et al.*, 2010). Similar to a *C. pneumoniae* infection in humans, *C. muridarum* can also disseminate throughout the mouse following an IN infection (Jupelli *et al.*, 2008), potentially by using monocytes as a shuttle system (Rey-Ladino *et al.*, 2007). *C. muridarum* is also able to colonise and inflame cardiac tissue following IN infection, a contributory risk factor in the development of CVD (Fan *et al.*, 1999). Murine models of autoimmune diseases like Alzheimer's, MS and

reactive arthritis have also been established (Hough and Rank, 1988; Rank *et al.*, 1988b; Du *et al.*, 2002; Little *et al.*, 2004).

The ideal animal model for predicting a vaccines efficacy will also reflect the human necessity for different immune populations for protection. Humans and mice generate *Chlamydia*-specific T and B cells following a pulmonary infection. Antibodies in human sera from the first infection wane over 3 – 5 years (Patnode *et al.*, 1990), which may explain the high re-infection rates. Similarly, the presence of antibodies confer resistance against a *C. muridarum* respiratory tract infection in mice (Yang and Brunham, 1998). Memory $CD4^+$ Th cells secreting IFNγ can be detected in PBMC following a natural infection in humans (Benagiano *et al.*, 2003; Benagiano *et al.*, 2005; Carralot *et al.*, 2005; Bunk *et al.*, 2010) and these are the dominant lymphocytes driving immunity against a respiratory tract infection in the mouse model (Penttila *et al.*, 1998; Penttila *et al.*, 1999; Rothfuchs *et al.*, 2004; Kadkhoda *et al.*, 2010; Zhang *et al.*, 2010). Therefore, the mouse model accurately replicates the many diseases associated with a *C. pneumoniae* infection in humans and shares a similar dependence on the different arms of the adaptive immune response as required in humans to eradicate an infection and prevent pathology.

Protection against a chlamydial pulmonary infection requires both a Th1 cell-mediated and Th2-driven antibody response, therefore many experimental vaccines have adopted the use of Th1 and Th2 polarising adjuvant combinations (Skelding *et al.*, 2006; Cheng *et al.*, 2009; Sun *et al.*, 2009; Ralli-Jain *et al.*, 2011). Intramuscular and SC immunisation with the MOMP and CpG-1826/Montanide ISA 720, CpG-1826/Alum or CpG-CTB, Th1 and Th2 polarising adjuvants respectively, produced a high level of systemic *Chlamydia*-specific antibodies and IFNγ-secreting T cells (Pal *et al.*, 2005; Cheng *et al.*, 2009; Sun *et al.*, 2009; Ralli-Jain *et al.*, 2010). This response confers a considerable amount of protection against cachexia and the amount of recoverable *Chlamydia* from the lung homogenates following an IN infection (Skelding *et al.*, 2006; Cheng *et al.*, 2009; Sun *et al.*, 2009; Ralli-Jain *et al.*, 2010). Despite being delivered parenterally (IM and SC), these vaccines were able to convey a substantial amount of protection against a mucosal pathogen because protective immunity in the lungs requires a systemic as well as a mucosal immune

response (Cheng *et al.*, 2009; Sun *et al.*, 2009; Ralli-Jain *et al.*, 2010). However, the inclusion of a mucosal route of immunisation has been shown to improve effectiveness of systemically delivered vaccines (Ralli-Jain *et al.*, 2010). Animals immunised with the MOMP and CpG-1826/Montanide ISA 720 via the IM, SC and SL routes produced greater *Chlamydia*-specific antibodies and IFNγ-secreting T cells than immunisation via systemic routes alone, which lead to less weight loss and lower amounts of recoverable *Chlamydia* following an IN infection (Ralli-Jain *et al.*, 2010). This highlights the importance of generating a mucosal response in addition to a systemic response for optimal protection against a chlamydial respiratory tract infection. Intranasal immunisation with the MOMP together with the Th1/Th2 polarising adjuvant CT/CpG, elicits the required systemic response with the added feature of mucosal antibodies and IFNγ-expressing T cells in the lymph nodes draining the lungs (Skelding *et al.*, 2006), a trait not previously reported following systemic immunisation alone (Pal *et al.*, 2002; Cheng *et al.*, 2009; Ralli-Jain *et al.*, 2010). These animals were not only significantly protected against cachexia, but had almost no recoverable *Chlamydia* at the usual peak of infection (Skelding *et al.*, 2006). A similar response was also achieved using the same vaccine administered via the TC route (Skelding *et al.*, 2006), which largely eliminates the potential for adjuvant toxicity (Mutsch *et al.*, 2004). However, the use of the IN route for protection against air-borne pathogens is highly desirable due to a strong ability to induce immunity in the lungs (Brandtzaeg, 2009), therefore the demand for safe yet effective adjuvants that can be delivered the IN route is greater than ever.

The CTA1-DD adjuvant has proven safe in primate models via the IN route (Sundling *et al.*, 2008), as it does not accumulate in the CNS (Eriksson *et al.*, 2004). Combined with the highly conserved influenza antigen M2e-HBc and administered intranasally, CTA1-DD provided complete protection against lethal influenza challenge and significantly reduced morbidity in mice (De Filette *et al.*, 2006). Animals immunised IN with the chlamydial MOMP, adjuvanted with CTA1-DD, induce strong systemic and mucosal *Chlamydia*-neutralising antibodies (Cunningham *et al.*, 2009). However, the ability of CTA1-DD to confer protection against a respiratory tract infection and the associated disease is yet to be investigated.

In the previous chapter we quantified and compared the systemic and mucosal immune responses of two vaccines delivered by multiple routes. Here we assessed the ability of each vaccination strategy to induce protection against a live *C. muridarum* pulmonary infection. Protection was determined by the ability of each vaccination strategy to prevent infection and pathological damage.

MATERIALS AND METHODS

Timeline

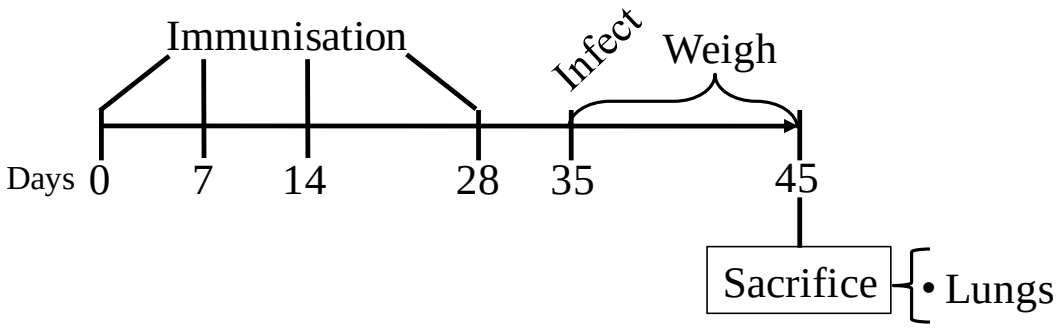


Figure 5.1: Experimental timeline for assessment of protection against a respiratory tract infection and pathology.

Mice were immunised on days 0, 7, 14 and 28 and infected intranasally 7 days after the final boost. Mice were weighed daily until sacrifice on day 45, 10 days post-infection (p.i). Lungs were collected for assessment of bacterial burden and disease.

Intranasal *C. muridarum* challenge and monitoring

Intranasal challenge was conducted as previously described (Skelding *et al.*, 2006). Briefly, 7 days after the final boost, animals were challenged intranasally with 10^3 IFU of purified *C. muridarum* in a 10µL volume, 5µL applied to each nare (Skelding *et al.*, 2006; Jupelli *et al.*, 2008; Ramsey *et al.*, 2009), as described for IN immunisation in the Chapter 3. Uninfected animals (no infection control) in addition to those recovering from a primary *C. muridarum* respiratory infection (live infection control), strongly resistant to re-infection, were also included as controls. All animals were weighed daily to monitor chronic inflammation and development of pneumonia indirectly through cachexia (Yang *et al.*, 1996). Percentage weight change was calculated by using the equation % weight change = [(body weight post-infection – body weight pre-infection) ÷ body weight pre-infection] × 100. All animals were euthanised on day 10 p.i at the peak of infectious burden and pathology for assessment of vaccine efficacy (Horvat *et al.*, 2010).

Quantification of chlamydial burden in the respiratory tract tissues

Quantification of recoverable amount of *Chlamydia* from lung homogenates was performed as previously described (Skelding *et al.*, 2006). The lungs from each mouse were excised and homogenised in 320µl of SPG using the OMNI TH tissue

homogeniser with the 7mm saw-toothed stainless steel attachment (OMNI International, Kennesaw USA). Total genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Cat No A1120, Promega, Alexandria, Australia) according to the manufacturer's instructions. Briefly, 50µL of tissue homogenate was digested overnight using proteinase K (Promega). DNA was then bound to a silica gel membrane, washed and finally eluted in nuclease-free water. DNA purify was assessed by the 260/280nm ratio using the ND-1000 nanodrop spectrophotometer (ThermoScientific, Wilmington, USA). Samples were stored at -80°C until required. Quantitative real-time PCR (qRT-PCR) was used to determine *C. muridarum* DNA levels from total mouse lung genomic DNA using a standard curve of the PCR product. The primer sequence used in the qRT-PCR amplified the outer membrane protein A (*omp*A) gene encoding the MOMP of *C. muridarum* is as follows: 5′-GCC GTT TTG GGT TCT GCT T-3′ and 5′-CGA GAC GTA GGC TGA TGG C-3′ (Sigma-Aldrich). Each reaction contained a final concentration of 1µM of forward/reverse primers, 200µM dNTP's, 1.5mM $MgCl_2$, 1X buffer, 0.15X SYBR green, and 5U of Platinum Taq polymerase (Invitrogen) made up to a final 20µL volume using sterile endonuclease-free water. Cycling conditions began with a standard 95°C for 20 sec, followed by a 64°C annealing for 20 sec and a 72°C extension for 15 sec repeated for a total of 30 cycles. qRT-PCR was performed using the Corbet Rotorgene Q (QIAGEN Pty Ltd, Chadstone centre, Australia).

Histopathology scoring

The lungs from each mouse were excised and preserved by fixing in 70%v/v ethanol. Tissues were then imbedded in paraffin, sectioned (5µm) and stained with Masson's trichrome (QML Diagnostics, Murarrie, Australia). Five random FOV of each section for each mouse were scored, by three individuals in a blinded fashion, based on fibrotic scaring of the lung tissue (Table 1.1). The final results were the average of all scores.

Table 5.1: Histopathological scoring system for mouse lungs

Category	Score	Description	Inflammation
Fibrosis	0	Lack of fibrosis around tissue	Absent
	1	Some fibrosis around tissue	Mild
	2	Some tissue has significant fibrosis Majority of tissue has some fibrosis	Moderate
	3	The majority of tissue has significant fibrosis	Severe

RESULTS

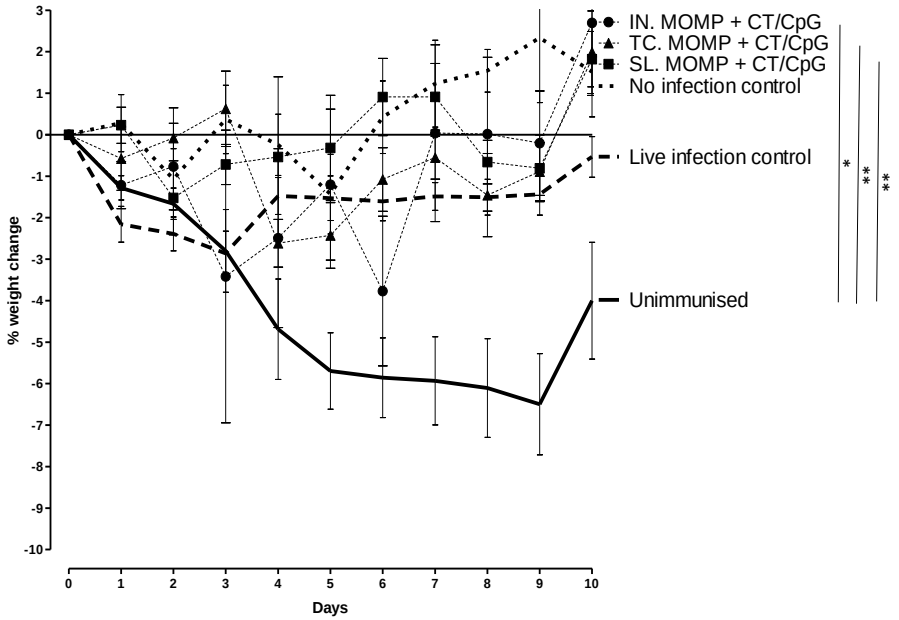
The effectiveness of each vaccine to protect against infection following live *C. muridarum* IN challenge

Cachexia

Cachexia is defined as a state of malnutrition, anorexia and muscle wasting (Emery, 1999). This type of weight loss is a commonly used marker of chronic inflammation and lung disease as it associates with increased concentrations of TNFα, IL-1β and neutrophil-attracting chemokines KC (CXCL1) and MIP-2 (CXCL2) in the BAL (van Heeckeren *et al.*, 2000). As live *Chlamydia* induce increased production of these cytokines and chemokines during an infection in a dose-dependent manner (Zaharik *et al.*, 2007; Zhang *et al.*, 2009; Zhou *et al.*, 2009; Rank *et al.*, 2010), cachexia can be used to monitor the development and clearance of pneumonia. Following an infection, unimmunised animals developed severe cachexia. The percentage weight loss of these naive animals peaked on day 9 p.i at approximately 7% (Figure 5.2). The live infection control animals, after recovering from a primary infection, were strongly protected against weight loss and hence re-infection. This group began regaining weight by day 4 p.i, significantly faster than the naive unimmunised animals at day 10 p.i (P <0.001). This signifies that the pneumonia that developed in the live infection control was relatively mild compared to that in the naive unimmunised animals. The weights of the no infection control animals fluctuated initially after the mock infection and then steadily increased from day 5. The no infection control group had a net positive weight gain over the 10 day time period, which was significant compared to infected control animals (P <0.001), indicating that cachexia was absent in uninfected animals. Immunisation with the CT/CpG-based vaccine significantly protected animals from weight loss regardless of the immunisation route (P <0.05 – 0.01) (Figure 5.2). The immunity elicited by the CT/CpG adjuvanted vaccine was comparable to that of the live infection control group. The route of immunisation influenced the protection elicited by the CTA1-DD-containing vaccine. Only IN delivery of the CTA1-DD-containing vaccine significantly prevented weight loss (P <0.05) when compared to the unimmunised control. The protection against pneumonia conferred by this vaccine was however equivalent to the strongly resistant live infection

control group. Neither SL nor TC immunisation with the CTA1-DD containing vaccine prevented the development of cachexia following infection.

A.



B.

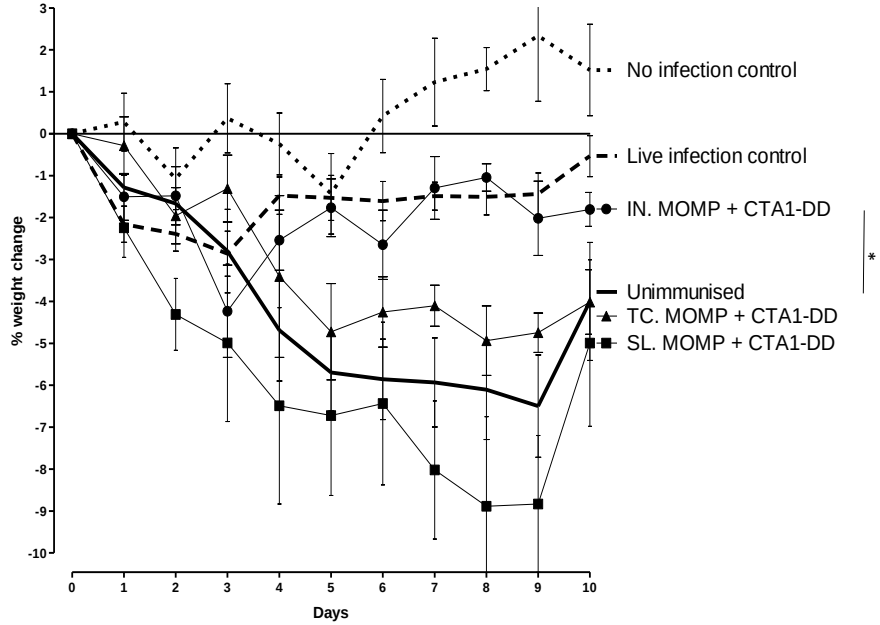


Figure 5.2: Percentage weight change of animals following IN challenge with *C. muridarum*.

Each mouse was weighed prior to infection to establish a starting weight and daily following infection to determine weight change. Percentage weight change was each animal was calculated by comparing the pre-infection body weight to daily p.i body weights. This figure depicts the effect each vaccination strategy has on weight change following an IN infection. (A) CT/CpG- or (B) CTA1-DD-based vaccines are grouped to show the effect the route of immunisation has on protection. Unimmunised (primary infection), live infection (secondary infection) and no infection controls are also included. Antigen (see below) and adjuvant controls were excluded from graphed data for simplicity (Appendix 3). Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test by comparing the weight changes between groups at the same point in time. One *P* value, the most significant, is given for groups showing a significant change. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

The protection against cachexia induced following IN immunisation was not significantly different between each vaccine or the live infection control, but weight loss was significantly lower in the vaccinated groups than the unimmunised controls (P <0.05) (Figure 5.3). However, the protection against weight loss elicited following IN immunisation with the MOMP antigen was similar, with or without an adjuvant.

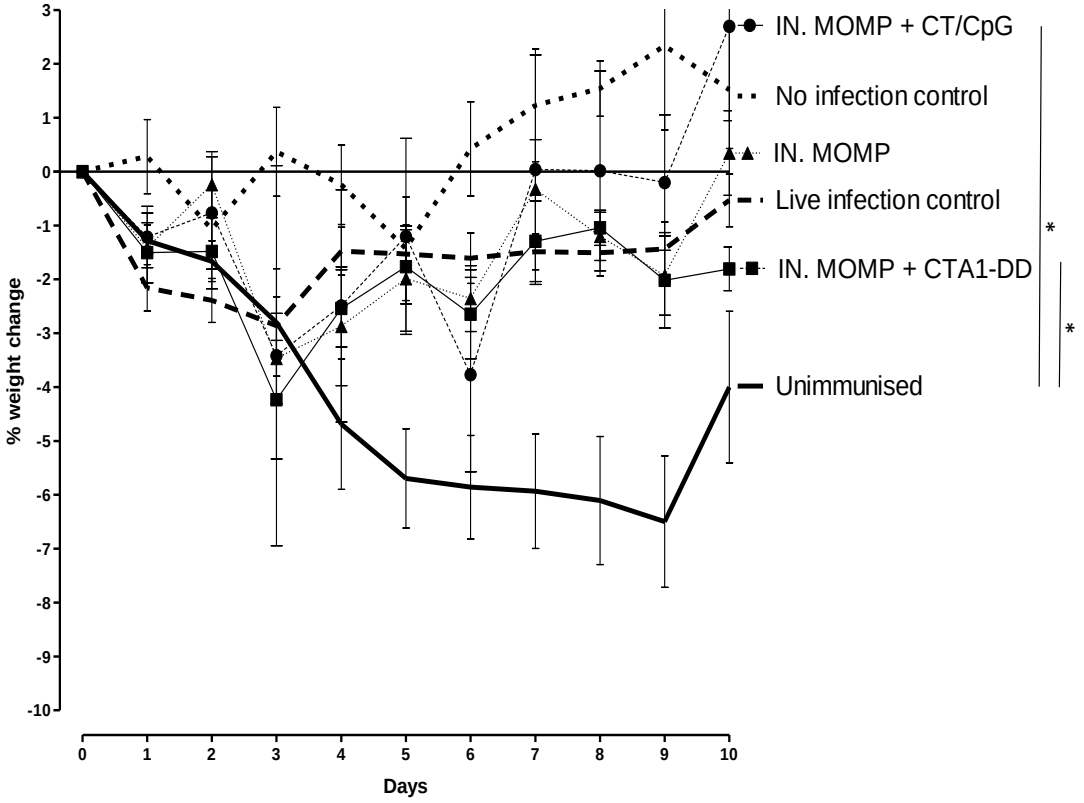


Figure 5.3: Percentage weight change of animals following IN challenge with *C. muridarum* following IN immunisation.

Each mouse was weighed prior to infection to establish a starting weight and daily following infection to determine weight change. Percentage weight change was for each animal was calculated by comparing the pre-infection body weight to daily p.i body weights. This figure depicts the effect each vaccination strategy has on weight change following an IN infection. Both CT/CpG- and CTA1-DD-based vaccines delivered via the IN route are grouped together to show the effect each adjuvant has on protection. Antigen, unimmunised (primary infection), live infection (secondary infection) and no infection controls are also included. Adjuvant controls were excluded from graphed data for simplicity (Appendix 3). Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test by comparing the weight changes between groups at the same point in time. One P value, the most significant, is given for groups showing a significant change. Significance was set at $P < 0.05$ for all tests. P >0.05 (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

Animals immunised with the SL delivered CT/CpG-based vaccine were the most protected against weight loss, losing a maximum of 2% of their initial weight before beginning to regain weight again by day 2 p.i (Figure 5.4). These mice were significantly protected from pneumonia when compared to the unimmunised (P <0.01) and antigen alone controls (P <0.05). Sublingual immunisation with the CTA1-DD adjuvanted vaccine provided no protection. Weight loss in this group actually exceeded that of the unimmunised controls.

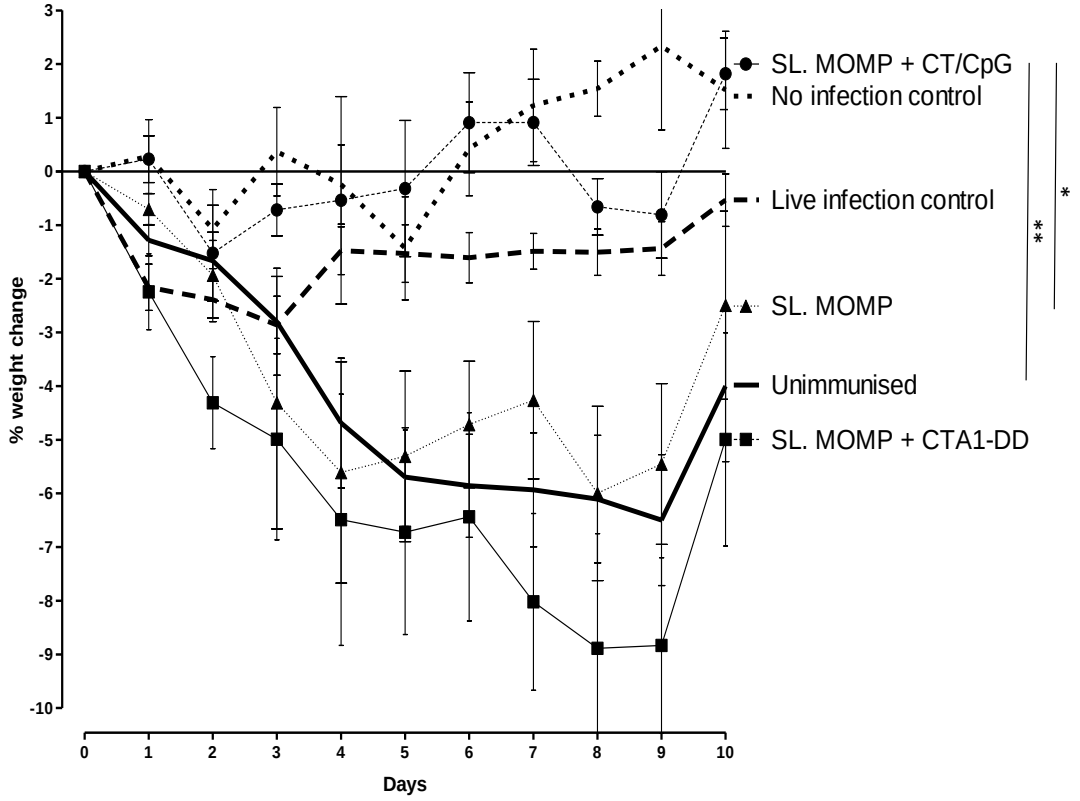


Figure 5.4: Percentage weight change of animals following IN challenge with *C. muridarum* following SL immunisation.

Each mouse was weighed prior to infection to establish a starting weight and daily following infection to determine weight change. Percentage weight change was for each animal was calculated by comparing the pre-infection body weight to daily p.i body weights. This figure depicts the effect each vaccination strategy has on weight change following an IN infection. Both CT/CpG- and CTA1-DD-based vaccines delivered via the SL route are grouped together to show the effect each adjuvant has on protection. Antigen, unimmunised (primary infection), live infection (secondary infection) and no infection controls are also included. Adjuvant controls were excluded from graphed data for simplicity (Appendix 3). Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test by comparing the weight changes between groups at the same point in time. One P value, the most significant, is given for groups showing a significant change. Significance was

set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

The immunity elicited by each vaccine following the TC immunisation was similar to the protection obtained following SL immunisation. Transcutaneous immunisation with the CT/CpG-containing vaccine provided robust protection against weight loss when compared to the unimmunised and antigen alone controls ($P < 0.01$). Minimal protection against weight loss was elicited by the CTA1-DD adjuvanted vaccine given by the TC route and was not statistically significant from unimmunised or antigen alone controls (Figure 5.5).

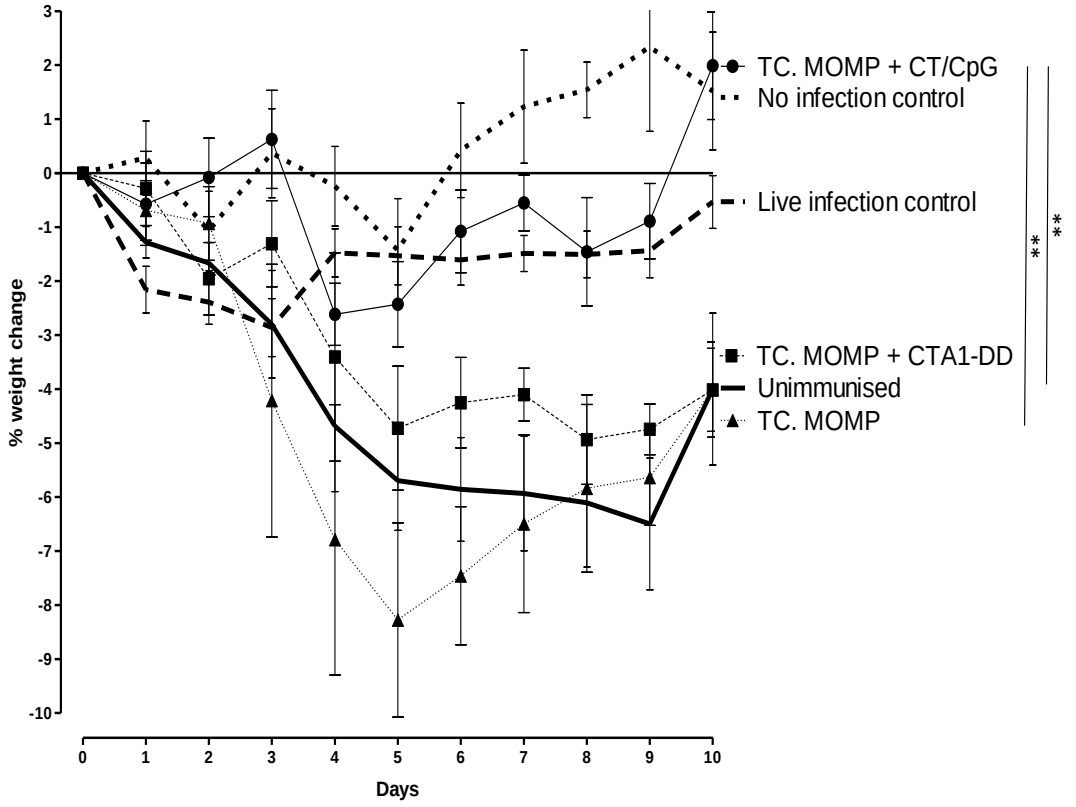


Figure 5.5: Percentage weight change of animals following IN challenge with *C. muridarum* following TC immunisation.

Each mouse was weighed prior to infection to establish a starting weight and daily following infection to determine weight change. Percentage weight change was for each animal was calculated by comparing the pre-infection body weight to daily p.i body weights. This figure depicts the effect each vaccination strategy has on weight change following an IN infection. Both CT/CpG- and CTA1-DD-based vaccines delivered via the TC route are grouped together to show the effect each adjuvant has on protection. Antigen, unimmunised (primary infection), live infection (secondary infection) and no infection controls are also included. Adjuvant controls were excluded from graphed data for simplicity (Appendix 3). Results are presented as the mean ± SD. Significant

differences were determined using a one-way ANOVA with Tukey's post-test by comparing the weight changes between groups at the same point in time. One *P* value, the most significant, is given for groups showing a significant change. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

Percentage weight change represented in the form of a line graph displays differences at the various time intervals. However, the net area under the curve analysis is a better depiction of the overall magnitude of cachexia over the duration of the infection. Unimmunised animals displayed a net negative weight change over the course of infection, indicating the development of severe pneumonia (Figure 5.6). Live infection control animals also had a net negative weight change, but this was significantly less than the naive unimmunised animals ($P < 0.01$), which indicates a more mild cachexia. No infection control animals had a net positive weight change, significantly different from infected controls ($P < 0.001$), indicative of a steadily increasing body mass. Both IN delivered vaccines were unable to reduce the overall magnitude of the cachexia that developed following infection when compared to the unimmunised control animals. Sublingual and TC immunisation with the CT/CpG adjuvanted vaccine significantly protected mice from cachexia when compared to the unimmunised controls ($P < 0.05$-0.01). Sublingual immunisation with the CT/CpG-based vaccines elicited a significantly greater level of protection against cachexia than the live infection control group ($P < 0.05$).

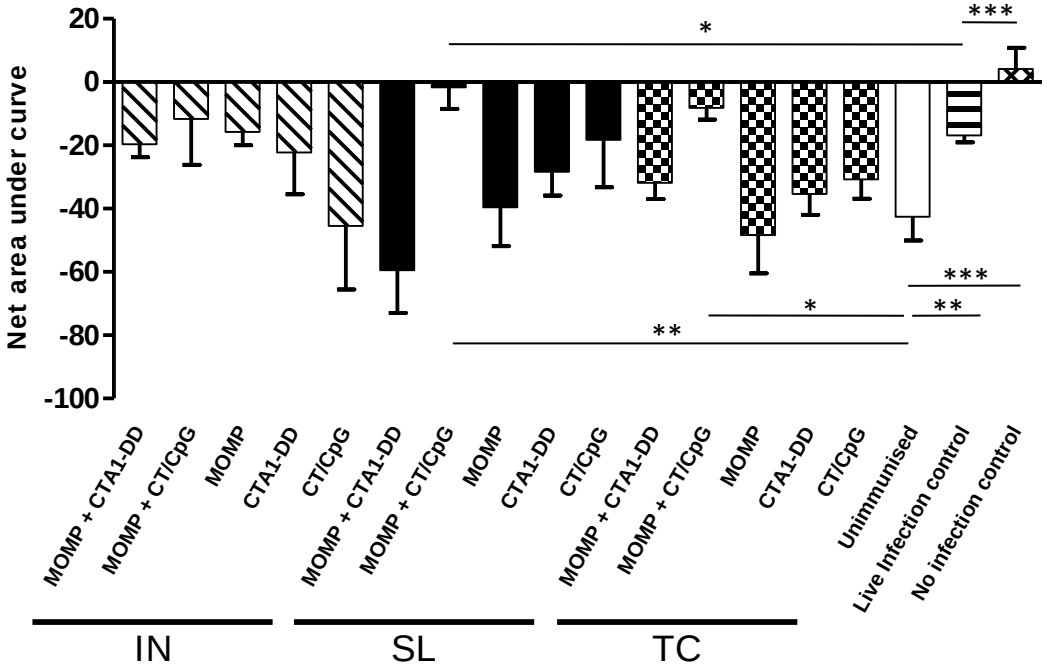


Figure 5.6: Net area under the curve of percentage weight change following IN challenge.

The area under the curve is a calculation of the region between the line of the percentage weight change and the x-axis (Appendix 3). This figure depicts the net total area, both negative and positive peaks, of body weight change over the 10 day course of infection in arbitrary units. Each vaccine was grouped with their respective route of immunisation, IN (▨), SL (■) and TC (▩). Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

Infectious burden

Preventing or even reducing the level of infection is crucial to control the transmission of infection. The peak of infection was assessed by quantifying the infectious burden in lungs on day 10 p.i at the height of a pulmonary infection in mice (Horvat *et al.*, 2007). This was used not only to measure the effect each vaccine had on the amount of recoverable *Chlamydia*, but also to validate cachexia results. Following infection, 100% of unimmunised animals were PCR positive for *C. muridarum* (Figure 5.7), with bacterial loads in excess of 4×10^4 copies/µg of genomic (g)DNA. All animals from the live infection control group were completely negative for chlamydial DNA at day 10 p.i. This abrogated course of infection in the live infection control group supports the strong resistance to re-infection indicated by the weight loss

data. The vaccines that were protective against cachexia also greatly reduced the bacterial burden in the lungs at the peak of infection. The CT/CpG-based vaccine significantly reduced levels of recoverable bacteria when compared to the unimmunised controls regardless of the immunisation route (P <0.05), whereas the CTA1-DD-containing vaccine was only protective following IN administration (P <0.05). Transcutaneous and SL immunisation with CTA1-DD adjuvanted vaccine, which failed to prevent the development of cachexia, also induced no significant reduction in total bacterial burden. The protection against bacterial burden elicited following IN immunisation with the MOMP antigen was similar, with or without an adjuvant, which verifies the weight loss data. No chlamydial DNA could be detected in the uninfected control group.

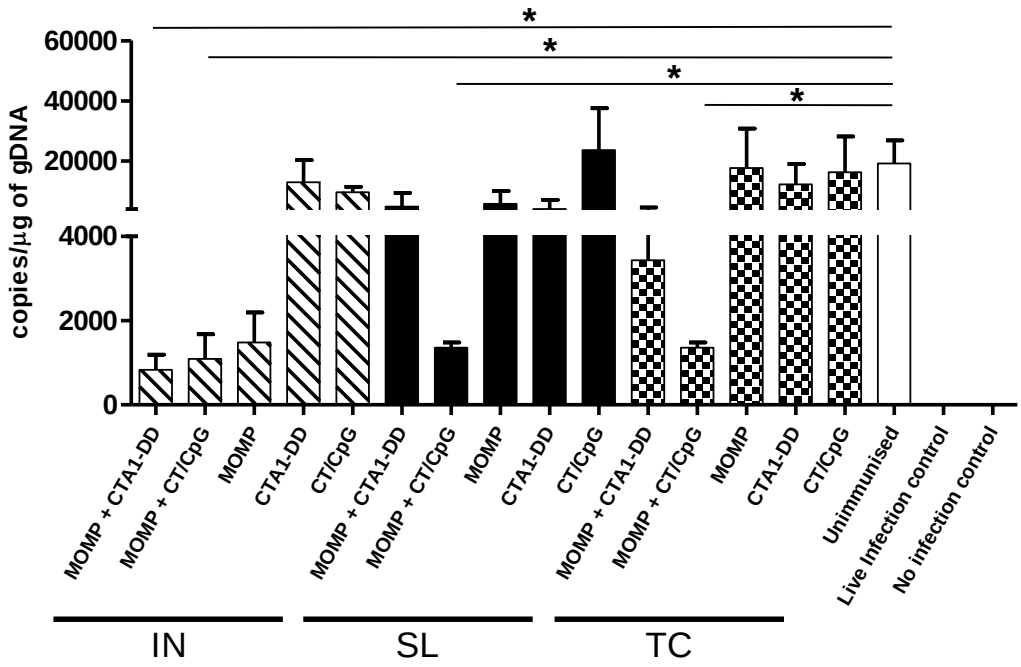


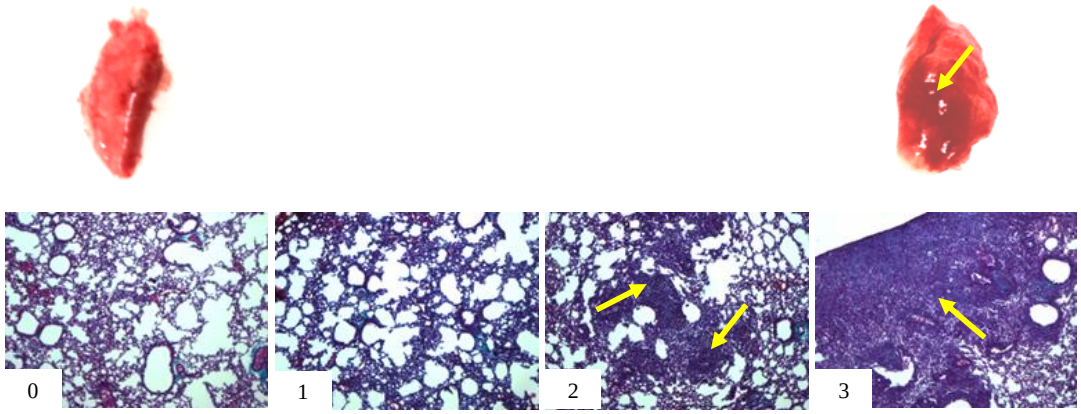
Figure 5.7: Chlamydial burden in lung tissue at day 10 p.i determined by *omp*A PCR.

Genomic DNA was extracted from lung tissues taken from all groups at day 10 p.i. Chlamydial DNA was quantified from lung gDNA by *omp*A-specific qRT-PCR using a standard curve. The copies of *C. muridarum* DNA per µg of host gDNA for each vaccine were grouped with their respective route of immunisation, IN (▨), SL (■) and TC (▣). Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at P < 0.05 for all tests. P >0.05 (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

The effectiveness of each vaccine to protect against the pathology that occurs following live *C. muridarum* IN challenge

While reducing the level of infection is important to minimise disease severity and transmission, a vaccine must also prevent the pathological changes associated with the exacerbation of certain chronic lung diseases. *C. muridarum* can reproduce many chronic inflammatory and autoimmune diseases in the mouse, associated with a *C. pneumoniae* infection in humans, although these often require the use of immunologically impaired mice ($IFN\gamma^{-/-}$) or unnatural route of infection (Hough and Rank, 1988; Rank *et al.*, 1988b; Fan *et al.*, 1999; Du *et al.*, 2002). We therefore determined whether each vaccine was capable of preventing the fibrotic scarring associated with an active chlamydial pulmonary infection (He *et al.*, 2010) and exacerbation of respiratory diseases like asthma and COPD (Postma and Timens, 2006). Pulmonary fibrosis, characterised by excessive growth of fibroblasts and extracellular matrix deposition (eg. collagen), is the main component of many lung diseases (Luzina *et al.*, 2008). Lung tissues taken from infected, unimmunised animals showed significant collagen deposition and lung consolidation, characteristics of a severe lung disease (Figure 5.8). The live infection control group, strongly resistant to re-infection, showed a similar level of fibrosis in the lungs to the naive unimmunised animals, as a result of the primary infection. The no infection control group displayed no evidence of fibrotic scarring when compared to the infection controls (unimmunised and live infection control) (P <0.001). Animals immunised with CT/CpG-based vaccines via the TC and SL routes that provided protection against cachexia and the bacterial burden, were also significantly protected against pathology when compared to the unimmunised controls. The greatest protection against fibrotic scarring however was seen in animals immunised by the TC route with the CTA1-DD adjuvanted vaccine, where the pathology score was equivalent to that in uninfected animals. Interestingly, these animals were not significantly protected against weight loss or bacterial burden. Neither vaccine delivered IN protected against pathology; in fact, both vaccines appeared to exacerbate fibrosis, despite reducing weight loss and bacterial burden.

A.



B.

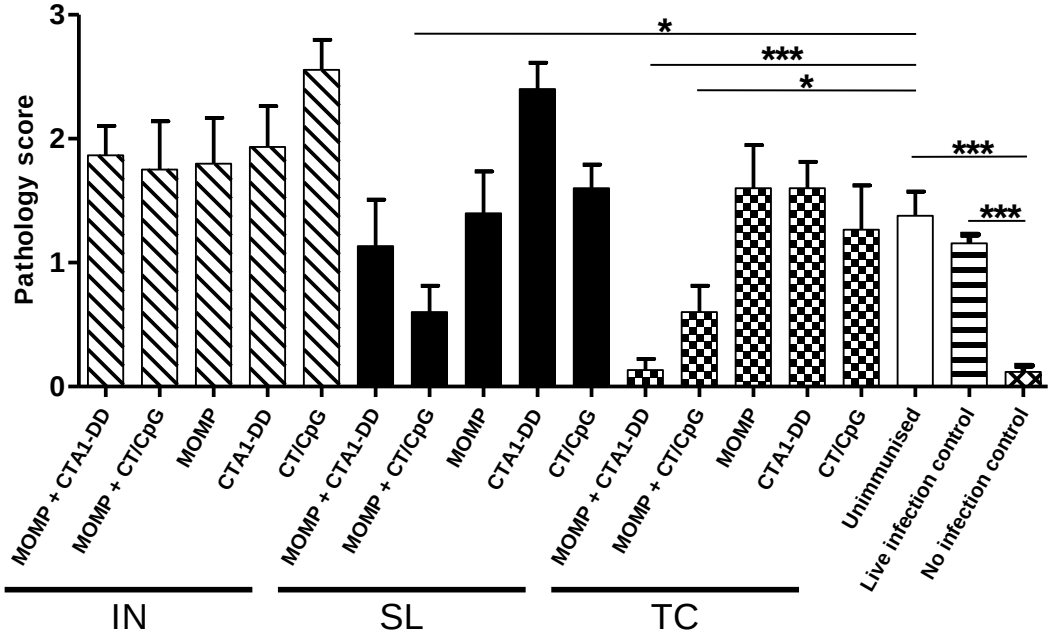


Figure 5.8: Fibrotic scarring and lung consolidation following IN infection with *C. muridarum*.

Lung tissues taken from each mouse at day 10 p.i were paraffin embedded, sectioned and stained for fibrosis using Masson's trichrome. (A) The pathology score scale used to compare the development of disease following infection. Representative histological images for each pathology scores are depicted. "0" signifies healthy, undamaged lung tissue. Scores of "1" through to "3" depict worsening degrees of collagen deposition (blue) and the beginnings of obstruction and consolidation of the lungs (yellow arrows). Pathology can also be seen on a macroscopic level, when comparing healthy (left) and unhealthy (right) lobes of the lungs. (B) Average pathology scores, assigned by three separate individuals blinded to groups and experimental design. Results are presented as the mean ± SD. Each vaccine is grouped with their respective route of immunisation, IN

(▨), SL (■) and TC (▩). Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

DISCUSSION

In this chapter, we assessed the level of protective immunity against a chlamydial respiratory tract infection and the associated disease generated following immunisation with two different vaccines, by a variety of needle-free routes. Immunisation of animals with the MOMP plus CT/CpG via the TC and SL routes significantly reduced infection-induced weight loss and the amount of recoverable *Chlamydia* from the lungs at day 10 p.i. This protection was similar to the immunity acquired after a natural infection, with the addition of near complete protection from pathology as determined by fibrosis and lung consolidation. Where the CT/CpG-based vaccine prevented progressive weight loss and bacterial burden regardless of administration route, CTA1-DD was only effective against a pulmonary infection following IN administration. Induction of strong serum IgG and splenic antigen-specific cellular responses were associated with the protection against infection, while local pro-inflammatory cells in the lymph nodes draining the lungs determined whether pathology was prevented (Chapter 4). Interestingly, the protection against infection elicited by both IN delivered vaccines (reduced weight loss and bacterial load) did not however, provide protection against pulmonary fibrosis. Moreover, TC immunisation with the CTA1-DD-based vaccine prevented pathology despite having no significant influence on chlamydial burden and only a minimal effect on weight loss. Therefore, this study not only identified a vaccine that protects against both infection and pathology, but also infection or pathology individually. This illustrates the challenges involved when developing a vaccine against *Chlamydia*: should the primary aim be to prevent infection (sterilising immunity) or prevent disease-associated pathology, as preventing/reducing infection does not necessarily protect against pathology and vice versa, at least in this mouse model.

Protection against chlamydial respiratory tract infection was associated with the induction of antibody and cell-mediated responses. *Chlamydia* has a unique biphasic lifecycle, consisting of the intracellular replicating RBs and the extracellular and infectious EBs. Effective protection against acute chlamydial infection elicited by MOMP-based vaccines requires the combination of Th1 cellular and Th2-driven antibody responses, targeting the RB and EB forms, respectively (Farris *et al.*, 2010).

The vaccines that induced a significant amount of protection against a respiratory tract infection, determined by weight loss and bacterial burden (Figure 5.7), generated the highest titres of MOMP-specific serum antibodies (Figure 4.9). In the absence of B cells and hence antibodies, mice resolve a primary and secondary genital tract infection similar to WT mice, however, these same mice are significantly more susceptible to re-infection (Su *et al.*, 1997). Similarly, B cell-deficient mice are more susceptible to re-infection following an IN chlamydial challenge, but also have higher mortality rates and develop more severe cachexia compared to WT mice (Williams *et al.*, 1997; Yang and Brunham, 1998). This suggests that antibodies prevent the establishment of an infection and control infection severity in the respiratory tract model. Although serum antibodies have been shown to neutralise *Chlamydia in vitro* and *in vivo* (Zhang *et al.*, 1987; Zhang *et al.*, 1989; Peterson *et al.*, 1993; Peterson *et al.*, 1997; Peterson *et al.*, 1998; Pal *et al.*, 1999), their contribution towards immunity against infection is not without contradiction. Many of the inconsistencies regarding the effect of serum antibodies in eradicating a chlamydial infection come from studies on the genital tract model of infection (Rank *et al.*, 1979; Ramsey *et al.*, 1988; Ramsey *et al.*, 1989; Buzoni-Gatel *et al.*, 1990; Cotter *et al.*, 1995; Morrison and Morrison, 2005), which is commonly reported to be more dependent on mucosal antibodies for protection. Conversely, systemic and mucosal transfer of convalescent serum confers protection against a subsequent *Chlamydia* respiratory challenge (Williams *et al.*, 1982; Williams *et al.*, 1984; Kaukoranta-Tolvanen *et al.*, 1995; Pal *et al.*, 2008). This difference between the responses required to elicit protection in the respiratory tract verses the genital tract is clear when we look at the protective effect of parenterally administered vaccines. Systemic immunisation with the MOMP and the adjuvant Alum, designed to elicit high serum antibody titres, confers a significant degree of protection against a respiratory tract infection but not following a genital tract challenge (Cheng *et al.*, 2011b; Yu *et al.*, 2012). This indicates that the induction of systemic antibodies can confer partial protection against a *Chlamydia* respiratory tract infection (Williams *et al.*, 1982; Williams *et al.*, 1984; Cheng *et al.*, 2009).

Titres of IgG and not IgA were a better associate of protection against a respiratory infection *in vivo*. The adoptive transfer of MOMP-specific IgG and IgA-producing

backpack hybridomas had a marginal affect on the clearance of a genital tract infection, yet IgG was significantly more protective against infection than IgA (Cotter *et al.*, 1995). Immunoglobulin A has also been shown to have no effect on the resolution of a primary respiratory tract infection or the incidence of mortality following passive immunisation (Williams *et al.*, 1984; Murthy *et al.*, 2004). This suggests that serum IgG may play a more dominant role than systemic IgA against a *Chlamydia* infection, supportive of our findings. However, the lack of protection induced by serum IgA *in vivo* may reflect an inability to elicit its primary function, which is direct neutralisation and prevention of microbial attachment to the mucosal surface (Janeway *et al.*, 2005; Cunningham *et al.*, 2008). Although the MOMP-specific IgA generated in the serum following vaccination significantly improved the neutralisation of *Chlamydia in vitro*, it may not have possessed the necessary secretory component required for receptor-mediated transport across the mucosal epithelium to allow IgA to neutralise the infection *in vivo* (Su *et al.*, 1995; Cunningham *et al.*, 2008). This hypothesis can be supported by the detection of low levels of IgA in the BAL (Figure 4.10), which did not induce a significant increase in neutralisation *in vitro* or coincide with protection against infection *in vivo* (Figure 5.2 – 5.7). Alternatively, IgG is able to passively transfuse from the serum into the highly vascularised lung mucosal tissues and potentially promote the Fc-mediated control of infection (Caldwell and Perry, 1982; Moore *et al.*, 2002). Therefore, IgA was ineffective at mediating protection against infection *in vivo*, possibly due to the induction of insufficient levels of IgA in the mucosal secretions needed to inhibit chlamydial attachment (Williams *et al.*, 1984). Induction of IgG antibodies was associated with protection from infectious challenge. The inability of IgG to neutralise *Chlamydia* infectivity *in vitro*, suggests that IgG may mediate protection *in vivo* through Fc-dependent mechanisms as opposed to direct neutralisation (Caldwell and Perry, 1982; Moore *et al.*, 2002).

Despite the positive influence high serum IgG titres had on protection against infection, the quantity, quality nor class of antibodies in the serum or mucosal secretions (Figure 4.9, 4.10) were associated with protection from pathology (Figure 5.8). Both IgG and IgA anti-MOMP antibodies can prevent the development of upper reproductive tract pathology following a genital tract infection (Cotter *et al.*, 1995).

Furthermore, IgA-deficient mice display exaggerated lung histopathology following an IN infection, even in the presence of a compensatory over-production of other serum antibodies (IgM, IgG, IgG2a, IgG1 and IgG2b) (Murthy *et al.*, 2004). This suggests that IgG is ineffective at preventing pathology, while IgA plays a role in regulating pulmonary inflammation and mucosal homeostasis following a primary chlamydial respiratory infection. The low levels of MOMP-specific IgA generated in the BAL following vaccination, which failed to neutralise the infection *in vitro* and elicit protection against infection *in vivo*, may have also been unable to prevent pathology. Moreover, suboptimal doses of IgG have even been associated with enhancement pathology (Peterson *et al.*, 1993; Su *et al.*, 1995; Peterson *et al.*, 1997; Cunningham *et al.*, 2011). The induction of MOMP-specific antibodies following vaccination was therefore unable to prevent the development of pathology after an IN challenge, despite their positive influence on infection.

The vaccines that elicited a significant level of protection against weight loss and infectious burden following an IN infection (Figure 5.2 – 5.7), induced secretion of the pro-inflammatory cytokine TNFα and T cell-related cytokines (IL-17 and IFNγ) by splenocytes following *in vitro* re-stimulation with the MOMP (Figure 4.4). These same vaccines also elicited low level secretions of the anti-inflammatory cytokines IL-4 and IL-10 by splenocytes (Figure 4.4). As the induction of pro-inflammatory cytokines (IFNγ, TNFα and IL-17) by T cells is known to enhance the resolution of infection (Williams *et al.*, 1993; Williams *et al.*, 1997; Lu and Zhong, 1999; Yang *et al.*, 1999; Jupelli *et al.*, 2008; Bai *et al.*, 2009; Zhang *et al.*, 2009; Jupelli *et al.*, 2010) and anti-inflammatory cytokines (IL-4 and IL-10) inhibit the clearance of a chlamydial respiratory tract infection (Williams *et al.*, 1997; Yang *et al.*, 1999), these vaccines primed the ideal response necessary to eradicate an active infection.

Pro-inflammatory cytokines have been shown to act individually or synergistically to improve inhibition of chlamydial growth. Regardless of its cellular source (Rothfuchs *et al.*, 2004), IFNγ is consistently shown to be crucial in the resolution of an active chlamydial infection. Although it is still to be determined whether IFNγ has a direct bacteriostatic or bactericidal effect on *C. muridarum* by inducing IRGs (Nelson *et al.*, 2005b; Coers *et al.*, 2011) or if protection is mediated by stimulating a

unique T cell population (Cotter *et al.*, 1997b). The Th17 cytokine IL-17 also confers protection against intracellular bacteria including *Chlamydia*, predominantly through the regulation of the Th1 response and neutrophil recruitment (Bai *et al.*, 2009). TNFα also positively influences the rate of bacterial clearance in the lungs (Williams *et al.*, 1990), through the recruitment and activation of numerous innate an adaptive populations. However, IFNγ-mediated inhibition of *Chlamydia* growth *in vitro* is enhanced 2-fold with the inclusion of TNFα (Summersgill *et al.*, 1995). The cytokine TNFα can increase expression of IFNγR and other transcriptional activators of IFNγ, thereby promoting IFNγ-mediated host defences (Robinson *et al.*, 2003). It has been suggested that the synergism between IFNγ and TNFα could be heightened further *in vivo*, as TNFα-mediated inhibition of chlamydial growth is indirect and requires the recruitment of additional cell populations (Perry *et al.*, 1999b). The cytokine TNFα also facilitates the release of IFNγ-induced NO by macrophages (Chen *et al.*, 1996; Igietseme, 1996; Fichorova and Anderson, 1999; Robinson *et al.*, 2003; Miljkovic and Trajkovic, 2004; Gabr *et al.*, 2011), which is important for infection resolution in the lungs (Qiu *et al.*, 2008). Together, TNFα and IFNγ increase expression of MHCII molecules and synergise with IL-17 to up-regulate ICAM-1 (Fichorova and Anderson, 1999; Gabr *et al.*, 2011), associated with immune cell recruitment, activation of Th1 cells and the normal resolution of a genital tract infection (Igietseme *et al.*, 1996; Igietseme *et al.*, 1999). Although we did not screen the type of cells secreting cytokines or assess for multi-functional phenotypes, emphasis has recently been placed on the contribution of $CD4^+$ T cells secreting a combination of cytokines ($IFN\gamma^+TNF\alpha^+$ and $IL\text{-}17^+IFN\gamma^+$ double positives, specifically) towards protection against a genital tract infection (Igietseme *et al.*, 1993; Olsen *et al.*, 2010; Yu *et al.*, 2010; Yu *et al.*, 2011; Yu *et al.*, 2012). This is because multi-functional T cells secrete larger amounts of cytokines and provide better co-stimulation for both T and B cells than single cytokine producing cell types (Kannanganat *et al.*, 2007). Infection with live EBs opposed to immunisation with dead EBs is known to induce stronger protection against re-infection, due in part to the generation of higher percentages of multi-functional T cells (Yu *et al.*, 2011). T cells co-producing multiple cytokines have been generated following vaccination and shown to confer a considerable amount of protection against a *Chlamydia*, *Mycobacterium tuberculosis* and *Leishmania major* (Darrah *et al.*, 2007; Forbes *et al.*, 2008; Kamath *et al.*, 2009;

Olsen *et al.*, 2010; Yu *et al.*, 2010; Yu *et al.*, 2012). Therefore, the induction of IFNγ, TNFα and IL-17 following immunisation coincided with strong protection against infection, potentially due to a synergistic interaction between multiple pro-inflammatory cytokines. To the best of our knowledge this is the first evidence of this combination cytokine response, first mentioned by Igiesteme *et al.*, (1993) and Yu *et al.*, (2010), conferring protection against *Chlamydia* in the respiratory tract infection model. We cannot definitively say whether $CD4^+$ T cells were co-producing these cytokines upon re-stimulation with the MOMP. However, the quality of T cells and the development of a multi-functional phenotype can be influenced by the adjuvant (Darrah *et al.*, 2007; Yu *et al.*, 2010; Yu *et al.*, 2012), duration of antigen exposure (Iezzi *et al.*, 1998; Jelley-Gibbs *et al.*, 2005), the type of APCs targeted (Reis e Sousa, 2004; Steinman and Hemmi, 2006) and the innate cytokine milieu at the site of immunisation (O'Garra, 1998)

Unexpectedly, we were unable to detect an IFNγ response in the spleen (Figure 4.4) or MdLN (Figure 4.6) following SL immunisation with the MOMP plus CT/CpG, even though this group of animals was significantly protected against infection. Interferon-γ was secreted by MOMP-stimulated lymphocytes isolated from the MiLN (Figure 4.8), but it is unlikely that these cells would have been activated rapidly following a pulmonary infection. It is possible that IFNγ-secreting MOMP-specific cells were present, but may have adopted a more effector phenotype migrating to other non-lymphoid tissues (Zhao *et al.*, 2003) or simply, the lymphocytes produced IFNγ following antigenic stimulation at a point in time not chosen for cytokine analysis (Han *et al.*, 2011).

Our data indicated that protection against infection was associated with a strong pro-inflammatory response in the spleen. Although TNFα and IFNγ are potent inhibitors of fibrosis *in vitro* and *in vivo* (Atamas and White, 2003; Pochetuhen *et al.*, 2007), the production of these cytokine by splenocytes could not be linked with protection against pathology. However, when a significant level of local antigen-specific proliferation and cytokine production was detected in the MdLN draining the respiratory tract (Figure 4.6), protection against infection (Figure 5.2 – 5.7) increased significantly and extended to include the prevention of pathology (Figure 5.8).

Activation of T cells occurs exclusively in the lymph nodes draining the lungs in the first few days following a pulmonary infection with a number of different viral and bacterial pathogens (Chackerian *et al.*, 2002; Lawrence and Braciale, 2004). Once activated, these T cells have been shown to undergo multiple rounds of proliferation and recruitment to the infected lungs, all of which can occur before an antigen-specific response can be detected in the spleen (Lawrence and Braciale, 2004). Furthermore, lymphotoxin-$\alpha^{-/-}$ (LTα) mice, which are completely deficient in lymph nodes and have altered splenic architecture, have an effective but delayed clearance of murine gammaherpes 68 virus and influenza virus infections, due to a decreased ability to initiate an immune response (Lee *et al.*, 2000; Lund *et al.*, 2002). This indicates the importance of lymph nodes when orchestrating rapid accumulation of T cells to the site of infection.

The enhancement of protection against infection and pathology seen in these groups may reflect the ability of antigen-specific cells residing in the lymph nodes draining the site of infection to respond more rapidly than the spleen-resident cells. Support for this hypothesis is provided by our findings that animals that mounted a local response in the lymph nodes draining the site of infection following vaccination (TC or SL delivered CT/CpG-based vaccine), were more resistant to weight change during the course of infection compared to animals that only mounted a systemic response when the same vaccine was delivered by the IN route. This has also been found to be the case for *M. tuberculosis*, where induction of T cells in the airways and not the spleen dictated protection from infection (Santosuosso *et al.*, 2005; Forbes *et al.*, 2008). In the context of protection against pathology, a reduction in the severity of lung histopathology following a *Burkholderia pseudomallei* infection has been associated with the early recruitment of inflammatory immune cells into the lungs (Judy *et al.*, 2012). Therefore, rapid activation and recruitment of a pro-inflammatory response to the lung mucosa may prevent adverse pathology, whereas a systemic response may be initiated following the recruitment of damaging innate populations to the lungs and the onset of pathology. Protection against pathology following an IN challenge with *Chlamydia* may rely on a vaccine priming and positioning antigen-specific cells in the regional lymph nodes, that can respond early during an infection and limit the involvement of the innate response in eradicating

the infection. To our knowledge this is the first evidence of the importance of generating a response in the lymph nodes following vaccination for protection against a chlamydial respiratory tract infection.

Interestingly, there appeared to be a significant disconnection between infection and pathology in some vaccine groups, where a reduction in bacterial burden did not necessarily coincide with protection against inflammatory disease and vice versa. Both IN delivered vaccines elicited protection from infection (Figure 5.2 – 5.7), yet seemed to exacerbate pathology compared to that of a normal course of infection (Figure 5.8). Intranasal immunisation elicited the strongest pro-inflammatory cytokine response that may have caused an imbalance between the control of infection and prevention of disease, resulting in excessive immunopathology (Lu *et al.*, 2011). Alternatively, it is conceivable that trace amounts of the vaccine may have drained into the lungs (Visweswaraiah *et al.*, 2002) and caused local inflammation and fibrosis, interpreted as infection-induced pathology.

This does not appear to be the case for the TC delivered CTA1-DD-adjuvanted vaccine group, which was solidly protected from pathology (Figure 5.8) despite having no significant reduction in the infectious burden (Figure 5.2 – 5.7). This disconnection between infection and pathology has two possible explanations; either (1) the development of pathology was merely postponed (Fraga *et al.*, 2012) or (2) the disease process has been circumvented in some way, independent of reducing the bacterial load. Defence against a pathogen has been proposed to occur via two mechanisms, either by limiting the pathogen burden (resistance) or the disease severity (tolerance) (Raberg *et al.*, 2007). The immunity induced by this particular vaccine appears to be one of tolerance against disease, as by definition, resistance has an effect on pathogen burden where tolerance does not (Raberg *et al.*, 2007). Tolerance to disease is not to be confused with immunological tolerance, which involves the suppression of antigen-specific responses, but instead describes a decreased susceptibility to immune- or pathogen-induced damage (Medzhitov *et al.*, 2012). However, tolerance to disease in some regards may refer to immunological tolerance. This concept of tolerance towards infection-induced disease was first described in animals by Råberg *et al*., (2007), where genetic differences were found

to influence tolerance to malaria, independent of parasitic load. Infection with *Plasmodium chabaudi chabaudi* causes haemolysis of red blood cells and results in a lethal form of hepatic failure. Expression of haeme oxygenase-1 (HO-1) catalyses the circulating free haeme, produced during haemolysis, preventing TNFα/ROS-mediated apoptosis by hepatocytes and hence liver failure (Seixas *et al.*, 2009). Mice expressing sickle haemoglobin are remarkably resistant to severe malaria, irrespective of the parasite load, as sickle haemoglobin not only prevents the expansion of pathogenic $CD8^+$ T cells but also induces expression of HO-1 in hematopoietic cells and prevents the cytotoxic effects of free haeme (Ferreira *et al.*, 2011). Even though this particular example is of a genetic predisposition driving tolerance against disease manifestation, it has been suggested that similar inducible protective pathways could exist for other types of infection, including *Chlamydia* (Miyairi *et al.*, 2012). Identifying and understanding these pathways may form the basis for new treatment strategies (Medzhitov *et al.*, 2012), that could include vaccines.

The type of immune response induced or the vaccine constituents may provide clues to what mechanism(s) may be involved in the development of tolerance against pulmonary fibrosis. Notably, this vaccine induced a strong IL-17 response in the spleen and MdLN following *in vitro* re-stimulation with the MOMP (Figure 4.4, 4.6), indicative of an elevated Th17 cell response. T cells can influence the fibrotic process in a number of ways, but are thought to function primarily in regulation of the healing process (Luzina *et al.*, 2008). The IL-17 cytokine has no direct effect on fibroblast proliferation or fibrosis (Molet *et al.*, 2001), but is a strong inducer of the anti-fibrotic Th1 response (Bai *et al.*, 2009). In the context of a *Chlamydia* pulmonary infection, IL-17 has been associated with the susceptibility to infection in C3H/HeN mice (Zhou *et al.*, 2009), yet neutralisation of IL-17 in BALB/c mice increases bacterial growth, weight loss and pathological changes (Bai *et al.*, 2009; Zhang *et al.*, 2009). Although, IL-17-mediated inflammation appears to prevent *Chlamydia*-induced immunopathology in the lungs, this involves limiting the pathogen burden by resistance as opposed to tolerance, contrasting to our findings.

The CTA1-DD adjuvant has also been implicated in the generation of antigen-specific tolerance. Fusion of the enzymatically inactive CTA1R7K-DD with H-2^q-restricted type II collagen peptide (amino acid 259–274), CTA1R7K-COL-DD, has been used to successfully treat collagen-induced arthritis in mice by inducing antigen-specific tolerance (Hasselberg *et al.*, 2009). CTA1R7K-COL-DD elicited immunological tolerance by the suppression of antigen-specific proliferation and IFNγ production following *in vitro* re-stimulation with collagen. However, this tolerogenic effect was only detected in the collagen-fused mutant devoid of enzymatic activity (CTA1R7K-COL-DD) and not the fully functional CTA1-COL-DD fusion. It is highly unlikely that the CTA1-DD used in the study was enzymatically inactive because it was fully functional and immunogenic via the IN route. Inactivation of the ADP-ribosyltransferase subunit of CTA1-DD by an interaction between the adjuvant and the permeation enhancer used to pre-treat the immunisation site is also improbable, as the skin was re-hydrated thoroughly prior to the vaccine being applied. Alternatively, other adaptive immune factors induced by vaccination and not assessed are limiting the fibrotic process or enhancing injury repair. This speculation requires further investigation that may well unveil yet undefined countermeasures against development of pathology.

CONCLUSIONS

The aim of this chapter was to assess the level of protective immunity against a *Chlamydia* respiratory tract infection and the associated disease following immunisation with the MOMP plus CTA1-DD or CT/CpG via TC, IN and SL routes. Aspects of the immune response induced following vaccination, determined in Chapter 4, were then associated with the level of protection against infection and disease. Induction of high serum IgG and not IgA associated with protection against infection and this was largely mirrored in the mucosal secretions. Systemic pro-inflammatory responses were also associated with the enhanced resolution of infection, possibly due to the synergistic effect of IFNγ, TNFα and IL-17 inhibiting chlamydial growth both directly and indirectly. Detection of an antigen-specific response in the MdLN draining the lungs associated with protection against pathology, which may reflect the time required to initiate and recruit an immune response to the site of the infection. However, protection against pathology developed in cases when the bacterial load recovered from the lung tissue was unaltered. This suggested that there may be other mechanisms modulating the development of disease following infection, which are independent of infection control.

CHAPTER SIX: PROTECTION AGAINST A REPRODUCTIVE TRACT INFECTION AND THE ASSOCIATED PATHOLOGY FOLLOWING AN INTRAVAGINAL CHALLENGE WITH *C. MURIDARUM*

INTRODUCTION

C. trachomatis (serovars D – K) is the most common bacterial STI worldwide, with over 250,000 new cases each day. Population prevalence is approximately 1 – 3% (Howie *et al.*, 2011; Ali *et al.*, 2012), but for high risk groups like adolescent women prevalence can reach almost 30% (Burstein *et al.*, 1998; Bunnell *et al.*, 1999; Cook *et al.*, 1999). Women also have the highest incidence of asymptomatic infection (between 70 – 90%) (Stamm and Holmes, 1990) and account for 80% of the total economic burden (Patel *et al.*, 2008). As the current strategy of detection and treatment is not only ineffective at controlling the prevalence of infection (Brunham *et al.*, 2005; Brunham and Rekart, 2008) and could in fact be contributing towards the uncontrolled spread of infection (Hadgu and Sternberg, 2009), a vaccine predominantly targeting females is recognised to have the greatest potential to impact on rates of infection and disease prevalence (Brunham *et al.*, 2005; Gray *et al.*, 2009).

Utilising an animal model that accurately reflects the many aspects of human infection and disease is vital for success in humans. Female mice can be infected via the vaginal vault with *C. muridarum*, the natural route of infection in humans (Ramsey *et al.*, 2009). This does however require an initial pre-treatment with progesterone (Tuffrey and Taylor-Robinson, 1981), which can alter the immunological balance of the mice to favour an anti-inflammatory response (Kita *et al.*, 1989). An IVag infection of mice leads to the colonisation of the vagina and a period of shedding of infectious chlamydial organisms. Shortly after the establishment of the infection in the lower reproductive tract, the infection ascends to colonise the upper reproductive tract (Carey *et al.*, 2009). This is followed by the development of hydrosalpinx, which is an accumulation of clear serous fluid in the oviduct (Carey *et al.*, 2009). Hydrosalpinx is characteristic of the scarring associated with tubal factor infertility in humans and the size of swelling of the oviduct is directly proportional to the extent of the damage (Shah *et al.*, 2005b; Imtiaz *et al.*, 2006). Blockages are thought to occur by scarring and adhesion of the oviducts and the disruption of the oviduct contractile activity responsible for clearing oviduct secretions and moving sperm to the uterus (Shibahara *et al.*, 2001; Dixon *et al.*,

2010). This pathology is hypothesised to be initiated and sustained by the pro-inflammatory cytokines/chemokines secreted by *Chlamydia*-infected epithelial cells, which in an ongoing chronic infection promote, cellular proliferation, tissue remodelling and scarring. Local production of IL-1β has been shown in an *ex vivo* model of Fallopian tubes infected with *C. trachomatis* to be a key initiator of direct tissue destruction, even in the absence of leukocytes (Hvid *et al.*, 2007). Similarly, IL-1β has also been implicated as a major driver of upper tract pathology in mice (Cheng *et al.*, 2008; Prantner *et al.*, 2009; Nagarajan *et al.*, 2012). However, these pathological effects of infection are also likely to be further exacerbated following the recruitment and infiltration of tissue-damaging innate and adaptive immune cell populations (Lee *et al.*, 2010b; Lu *et al.*, 2011).

In addition to replicating human disease, the ideal model for predicting a vaccine's efficacy will also imitate the reliance on different immune populations for protection. Humans and mice elicit *Chlamydia*-specific T and B cells are detectable in humans and mice following a genital tract infection (Mittal *et al.*, 1996; Morrison and Caldwell, 2002; Agrawal *et al.*, 2007). Antibodies contribute to immunity against re-infection in mice (Morrison and Morrison, 2005), although the role mucosal and systemic antibodies play in the resolution of a chlamydial genital tract infection in humans is unclear (Mittal *et al.*, 1996). Interferon γ-secreting $CD4^+$ Th1 cells can be detected in the female reproductive tract and in PBMC following a natural infection in humans (Mittal *et al.*, 1996; Agrawal *et al.*, 2007) and as with mice (Morrison and Caldwell, 2002), these were associated with protection against infection and disease in humans (Debattista *et al.*, 2002; Kinnunen *et al.*, 2002). Therefore, the mouse model accurately replicates the many facets of disease associated with a *C. trachomatis* infection in humans and shares a similar dependence on the different arms of the adaptive immune response required in humans to eradicate an infection and prevent pathology.

Protection against a chlamydial genital tract infection relies on the generation of Th1 cell-mediated response and to a lesser extent Th2-driven antibody response. As a result, the majority of chlamydial vaccines incorporate Th1 polarising adjuvants. Utilisation of the adjuvants CAF01, DDA-MPL, DDA-TDB, CpG-1826, CpG-

1826/IL-12, in conjunction with a multitude of different antigens, induced high levels of IFNγ-secreting Th1 cells (Li *et al.*, 2007; Yu *et al.*, 2010; Murthy *et al.*, 2011b; Yu *et al.*, 2012). This response confers partial protection against the vaginal shedding (Li *et al.*, 2007; Yu *et al.*, 2010; Murthy *et al.*, 2011b; Yu *et al.*, 2012) and oviduct pathology (Li *et al.*, 2007; Murthy *et al.*, 2011b). Induction of optimal protective immunity by MOMP-based vaccines however requires the contribution of both $CD4^+$ T cells and antibodies to prevent the establishment of infection (Farris *et al.*, 2010). The combination of the MOMP with Th2 as well as Th1 polarising adjuvants CT and CpG improves the induction of IFNγ-expressing T cells and mucosal antibodies, which associates with enhanced clearance of a chlamydial genital tract infection (Berry *et al.*, 2004). Furthermore, the use of mucosal and cutaneous routes of immunisation is known to improve the homing of antigen-specific responses to the genital tract via the CMIS (Czerkinsky and Holmgren, 2010a; Czerkinsky and Holmgren, 2010b). To utilise these routes however requires a potent adjuvant to overcome tolerance, which is commonly elicited towards an antigen when it is applied to the epithelium. Intranasal immunisation with the MOMP together with potent mucosal adjuvants CT and CpG, significantly reduced that amount of recoverable *Chlamydia* following a genital tract infection (Barker *et al.*, 2008). However, the toxicity of CT and CpG following IN administration precludes their use in human prophylactic vaccines. (Fujihashi *et al.*, 2002; Couch, 2004; Heikenwalder *et al.*, 2004; Kiyono and Fukuyama, 2004; Mutsch *et al.*, 2004; Klinman *et al.*, 2007; DeFrancesco, 2008). These adjuvants can also generate mucosal immunity by routes other than IN and confer a considerable amount of protection against a genital tract infection (Berry *et al.*, 2004; Huang *et al.*, 2008; Cuburu *et al.*, 2009). Most importantly, these routes limit the toxic side effects commonly affiliated with nasal delivery of potent mucosal adjuvants.

Utilisation of the IN route to elicit protection against mucosal pathogens is still highly desirable due to a strong ability to induce immunity in the genital tract (Brandtzaeg, 2009). Therefore, the demand for safe yet effective adjuvants that can be administered via the IN route is greater than ever. As CTA1-DD does not accumulate in the CNS (Eriksson *et al.*, 2004), it has been shown to be a non-toxic yet potent mucosal adjuvant (Sundling *et al.*, 2008). The CTA1-DD adjuvant

combined with the HIV-1 envelope glycoprotein (Env) induced a significantly greater amount of mucosal IgA when compared to the same vaccine given parenterally (Sundling *et al.*, 2008). Animals immunised IN with the chlamydial MOMP, adjuvanted with CTA1-DD, produce strong systemic and mucosal *Chlamydia*-neutralising antibodies, which enhanced the resolution of a *Chlamydia* genital tract infection (Cunningham *et al.*, 2009). However, it is unclear whether CTA1-DD can be used in conjunction with the MOMP by alternative immunisation routes, perhaps more capable of stimulating greater mucosal immunity against a genital tract infection and the associated disease.

Chapter 4 compared and quantified the systemic and mucosal immune responses of two vaccines delivered by multiple routes. In this chapter the ability of each vaccination strategy to induce protection against a live *C. muridarum* genital tract infection was assessed. Protection was determined by the ability of each vaccination strategy to reduce infection and pathological damage.

MATERIALS AND METHODS

Timeline

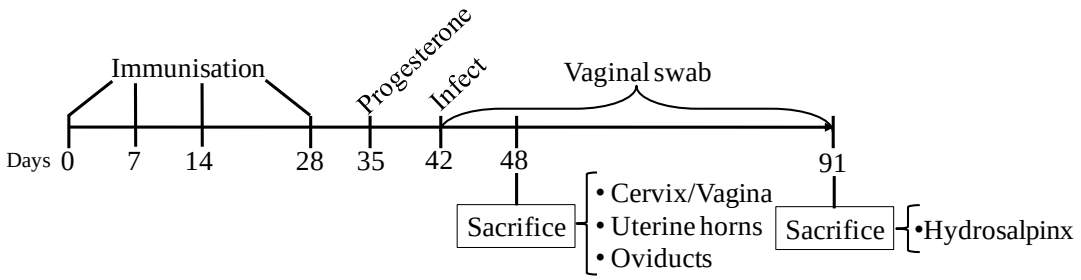


Figure 6.1: Experimental timeline for assessment of protection against a genital tract infection and pathology.

Mice were immunised on days 0, 7, 14 and 28 and progesterone treated 7 days prior to being infected intravaginally. Mice were sacrificed on day 48, 6 days p.i, to collected cervix/vagina, uterine horn and oviducts for assessment of bacterial burden and gene expression. A second group of identical animals was also culled on day 91, 49 days p.i, for analysis of oviduct upper reproductive tract disease. Vaginal swab were taken for both groups every 3 to 7 days following the IVag challenge.

Intravaginal *C. muridarum* challenge

Intravaginal challenge was conducted as previously described (Carey *et al.*, 2009). Each animal received a SC injection with 2.5mg of medroxyprogesterone (Depo Provera), 7 days prior to challenge with *C. muridarum*. Anesthetised mice were given 5 x 10^2 IFU of *C. muridarum* into the vaginal vault in 20µL of SPG. The genital region was swabbed after infection with 70%v/v ethanol to remove any *Chlamydia* from the exterior. Progesterone primed, uninfected animals (no infection control) in addition to those recovering from a primary *C. muridarum* genital infection (live infection control), strongly resistant to re-infection, were also included as controls.

Monitoring clearance of infection and burden

Vaginal swabs were taken by swabbing the vagina five times clockwise and anti-clockwise using a sterile nasopharyngeal Calgiswab (Modular Medical Products, Alice Springs, Australia) moistened with ice-cold SPG. Collection of vaginal swabs occurred every 3 days until day 21 p.i, from then every 7 days until sacrifice (day 49 p.i). Swabs were submerged in 500µL of SPG containing two sterile glass beads, and vortexed for 20 sec before storing at -80°C. Swabs were vortexed again after thawing

and before analysis. Cervico-vagina, uterine horn and oviduct tissues were excised and placed in 300µL of ice-cold SPG. Before storing at -80°C, tissues from the reproductive tract were homogenised (OMNI TH tissue homogeniser) at 5,000rpm until tissues were completely dispersed. Tissue homogenates were centrifuged at 500x*g* for 5 min at 4°C before storing and culturing. Infection was quantified using the tissue homogenate to infect a confluent monolayer and stained using the method previously (Chapter 3). Animals were deemed to have a productive infection at level ≥300IFU per swab (Carey *et al.*, 2009).

Assessment of oviduct pathology

Presence of hydrosalpinx was noted for gross pathology. Although the formation of hydrosalpinx is not a definitively representative of oviduct occlusion (Shah *et al.*, 2005b), its presence does suggest tubule damage and potential fertility complications. Hydrosalpinx was measured post mortem at day 49 p.i. Presence/absence of hydrosalpinx indicated the incidence of oviduct occlusion and the oviduct diameter reflected the severity of the blockage.

Ribonucleic acid (RNA) extraction and gene expression analysis

Oviducts were excised on day 6 p.i and stored in RNAlater (QIAGEN) at -80°C. Oviducts were separated from the ovary and uterine horns using a stereomicroscope (Leica-Microsysems, North Ryde, Australia). Total RNA was extracted from pooled oviduct homogenate using the RNeasy Fibrous Tissue mini kit (Cat No. 74704, QIAGEN) and treated with RNase-free DNase (QIAGEN) as per the manufacturer's instructions. The purity of RNA was assessed by the 260/280nm ratio using the nanodrop spectrophotometer (ThermoScientific). Complimentary DNA (cDNA) was synthesised from 1µg of total RNA using RT^2 First Strand synthesis kit (Cat No. 330401, QIAGEN) as per the manufacturer's instructions. cDNA samples were mixed with RT^2 SYBR ROX qPCR mastermix, aliquoted into the RT^2 Profiler Mouse Th17 and Autoimmunity PCR Array (Cat No. PAMM-073A SABioscience-QIAGEN), amplified and analysed on a 7900HT FAST ABI system (Applied Biosystems, Mulgrave, Australia). Expression was normalised using multiple reference genes (Glucuronidase-β – *gus*B, hypoanthine guanine phosphoribosyl – HPRT, HSP90ab1, Glyceraldehyde-3-phosphate dehydrogenase – GAPDH, β actin –

*act*B). Fold-changes were calculated using PCR Array Data Analysis Software 3.0 (QIAGEN) based on the $^{\Delta\Delta}$Ct method of relative quantification. Expression was further normalised against progesterone treated uninfected animals.

RESULTS

The effectiveness of each vaccine to protect against infection following live *C. muridarum* IVag challenge

A vaccine with the potential to reduce both the peak bacterial load as well as the duration of chlamydial shedding in the lower genital tract, could significantly reduce infection transmission (Gray *et al.*, 2009). We therefore determined the degree of protection induced by each vaccine by quantifying the amount of bacteria shed into the vagina following an IVag challenge with *C. muridarum* (Figure 6.2). The infection in the naive unimmunised group peaked at day 6 p.i and animals continued to shed *Chlamydia* into the vagina for a period of 35 days (Table 6.1). The live infection control mice, recovering from a primary infection, were strongly resistant to re-infection and were not found to have a productive infection (>300IFU) in the vagina. Although no vaccine was able to elicit an equivalent level of protection to the live infection control, against the establishment of an infection, IN immunisation with either vaccine significantly reduced the peak bacterial load in the vaginal shedding when compared to the unimmunised controls at day 6 p.i ($P<0.01$). Both vaccines administered via the SL route significantly decrease the bacterial load shed into the vagina ($P<0.05$), whereas only the CTA1-DD-based vaccine delivered by the TC route effectively reduced shedding at day 6 p.i ($P<0.05$).

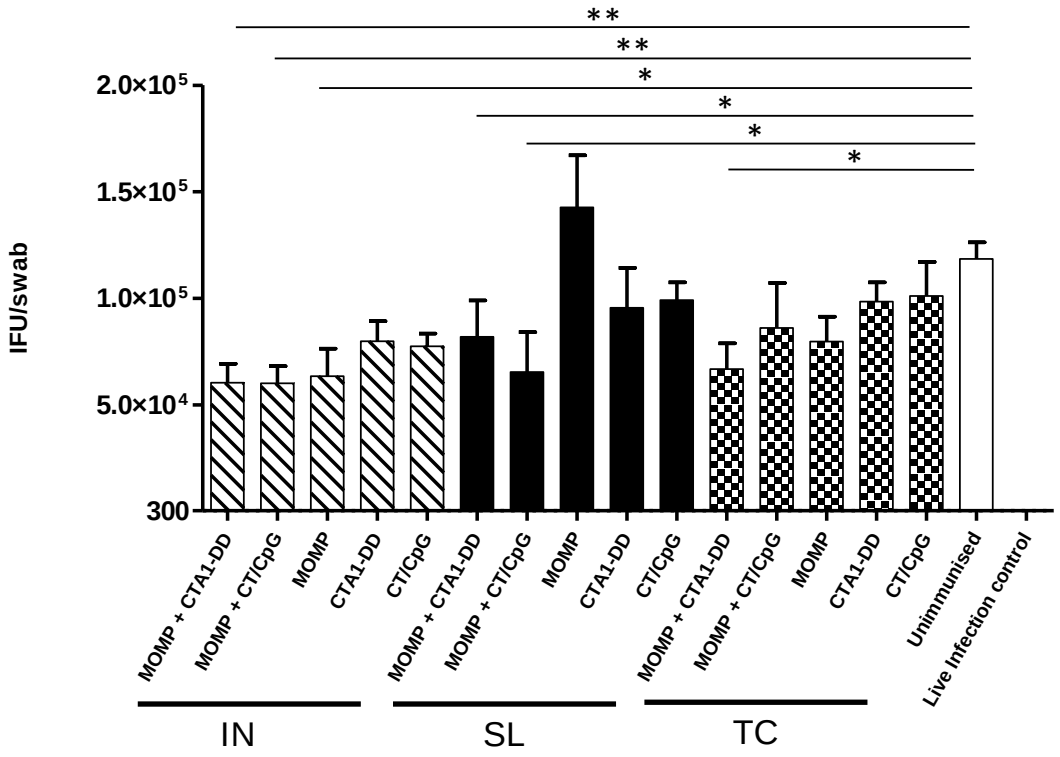


Figure 6.2: The *C. muridarum* burden in the vagina and collected by the vaginal swab at day 6 p.i.

Following an IVag challenge with *C. muridarum*, vaginal swabs were collected over the entire duration of an infection and stored in SPG. The amount of C. muridarum collected by each swab (IFU/swab) was quantified *in vitro* from the SPG. Cut-off level for a productive infection was set at ≥300IFU/swab. Results are presented as the mean ± SD. Each vaccine is grouped with their respective route of immunisation, IN (▨), SL (■) and TC (▩). Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

The duration of vaginal shedding was significantly decreased following SL and TC immunisation with CT/CpG-based vaccines ($P<0.01$) (Table 6.1). All of these vaccinated animals had ceased shedding by day 18 p.i compared to day 35 p.i for unimmunised animals, reducing the duration of infection in the lower genital tract by 50%. Intranasal immunisation with the CT/CpG containing vaccine also reduced the duration of shedding, although this was not significant from the adjuvant control group. Immunisation with the CTA1-DD adjuvanted vaccine failed to shorten the infection duration, regardless of the immunisation route.

Table 6.1: Number of animals with a detectable vaginal *C. muridarum* infection collected by vaginal swabbing.

	Days									
Group	3	6	9	12	15	18	21	28	35	
IN. MOMP + CTA1-DD	5/5	5/5	5/5	5/5	5/5	4/5	1/5	0/5	0/5	
IN. MOMP + CT/CpG	4/4	4/4	4/4	4/4	3/4	3/4	2/4	0/4	0/4	
IN. MOMP	5/5	5/5	5/5	5/5	5/5	4/5	1/5	0/5	0/5	
IN. CTA1-DD	5/5	5/5	5/5	5/5	5/5	4/5	1/5	0/5	0/5	
IN. CT/CpG	5/5	5/5	5/5	5/5	5/5	4/5	0/5	0/5	0/5	
SL. MOMP + CTA1-DD	5/5	5/5	5/5	5/5	4/5	3/5	2/5	0/5	0/5	
SL. MOMP + CT/CpG	5/5	5/5	5/5	4/5	2/5	0/5	0/5	0/5	0/5	***
SL. MOMP	5/5	5/5	5/5	5/5	4/5	3/5	1/5	0/5	0/5	
SL. CTA1-DD	5/5	5/5	5/5	5/5	4/5	3/5	1/5	0/5	0/5	
SL. CT/CpG	5/5	5/5	5/5	5/5	5/5	5/5	2/5	0/5	0/5	
TC. MOMP + CTA1-DD	5/5	5/5	5/5	5/5	5/5	5/5	1/5	0/5	0/5	
TC. MOMP + CT/CpG	5/5	5/5	5/5	5/5	1/5	0/5	0/5	0/5	0/5	***
TC. MOMP	5/5	5/5	5/5	5/5	5/5	5/5	4/5	0/5	0/5	
TC. CTA1-DD	5/5	5/5	5/5	5/5	5/5	5/5	4/5	1/5	0/5	
TC. CT/CpG	5/5	5/5	5/5	5/5	5/5	5/5	2/5	0/5	0/5	
Unimmunised	15/15	15/15	15/15	15/15	15/15	14/15	11/15	1/15	0/15	
Live infection control	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	***

Following an IVag challenge with *C. muridarum*, vaginal swabs were collected over the entire duration of an infection and stored in SPG. The amount of *C. muridarum* collected by each swab was quantified *in vitro* from the SPG. Animals were deemed to have productive infection at ≥300IFU/swab. Results are presented as number of animals with a productive infection as the numerator, over the total number of animals in each group as the denominator. The heat map represents 100% of animals infected in black, grey as animals begin to clear the infection and white when 100% of animals had no detectable infection. Significant differences were determined using a Kaplan-Meier survival curve and the log rank post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

The net area under the curve analysis of the course of vaginal shedding is a better depiction of the overall magnitude of the infection measured in the lower reproductive tract. Sublingual and TC immunisation with CT/CpG adjuvanted vaccine significantly reduced the overall magnitude of the infection in the vagina when compared to the unimmunised naive control group (Figure 6.3). Immunisation with the CTA1-DD containing vaccine was unable to significantly decrease the overall bacterial magnitude measured in the lower genital tract.

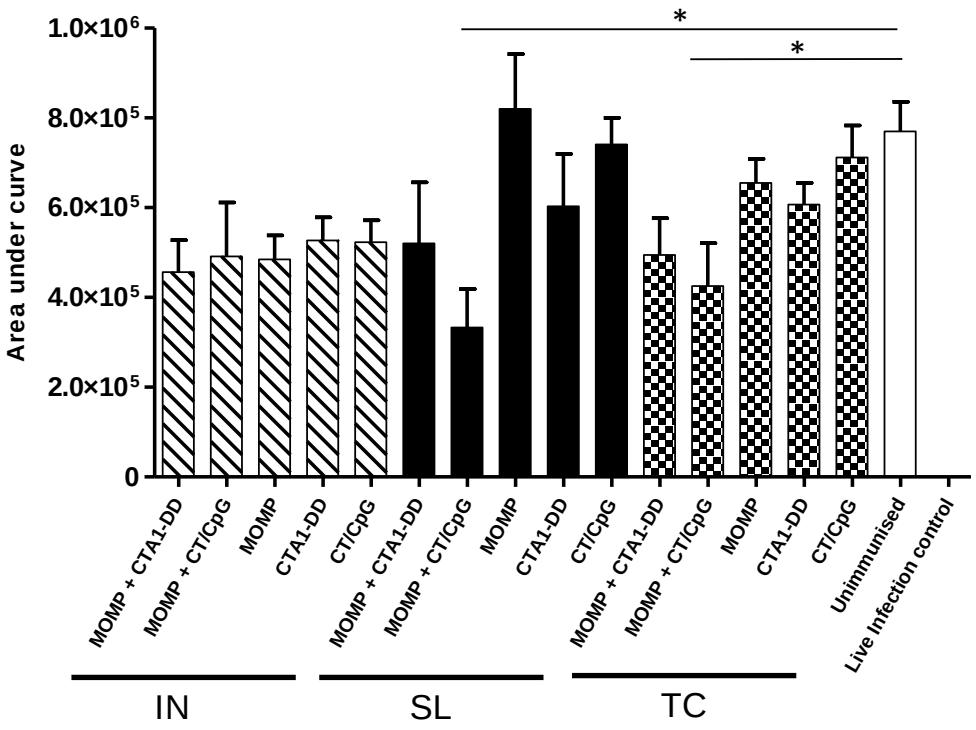


Figure 6.3: Infection magnitude determined by the net area under the curve of vaginal shedding.

The area under the curve is a calculation of the region between the line drawn for vaginal shedding (IFU/swab) and the x-axis (Appendix 4). This figure depicts the net total area of the vaginal shedding for each group over the entire course of infection in arbitrary units. Results are presented as the mean ± SD. Each vaccine is grouped with their respective route of immunisation, IN (▨), SL (■) and TC (▩). Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

Preventing the ascension of the infection from the vagina into the upper reproductive tract can prevent the development of pathology (Cotter *et al.*, 1995). We therefore determined the ability of each vaccine to prevent infection ascension and colonisation of the upper reproductive tract tissues at the peak of infection on day 6 p.i (Carey *et al.*, 2009). In the unimmunised naive animals, the greatest quantity of *Chlamydia* was detected in the cervico-vagina, collected by the vaginal swab (Figure 6.4). Proportionally, the oviducts contained the highest bacterial load of all tissues in unimmunised animals, followed by the cervico-vagina then the uterine horns. Low levels of infection were detected in the tissues of the live infection control group, which verifies the strong protection against re-infection indicated by the vaginal shedding data. Intranasal immunisation with the CT/CpG adjuvanted vaccine was the

only vaccine to significantly decrease the infectious burden in the upper reproductive tract at the peak of infection when compared to the unimmunised controls (P<0.01). In animals immunised with CT/CpG adjuvanted vaccine via the IN route, the infection either failed to ascend beyond the uterine horns or was eradicated from the upper reproductive tract by day 6 p.i. The protection against infection of the upper reproductive tract conferred by this vaccine matched with a decrease in the chlamydial shedding peak burden and duration measured in the lower reproductive tract (Figure 6.2; Table 6.1). Sublingual and TC immunisation with the CT/CpG-based vaccine, which significantly reduced the peak and duration of vaginal shedding, were however unable to prevent the colonisation of the upper reproductive tract. The CTA1-DD adjuvanted vaccine failed to prevent the ascension of the infection, regardless of the route of administration.

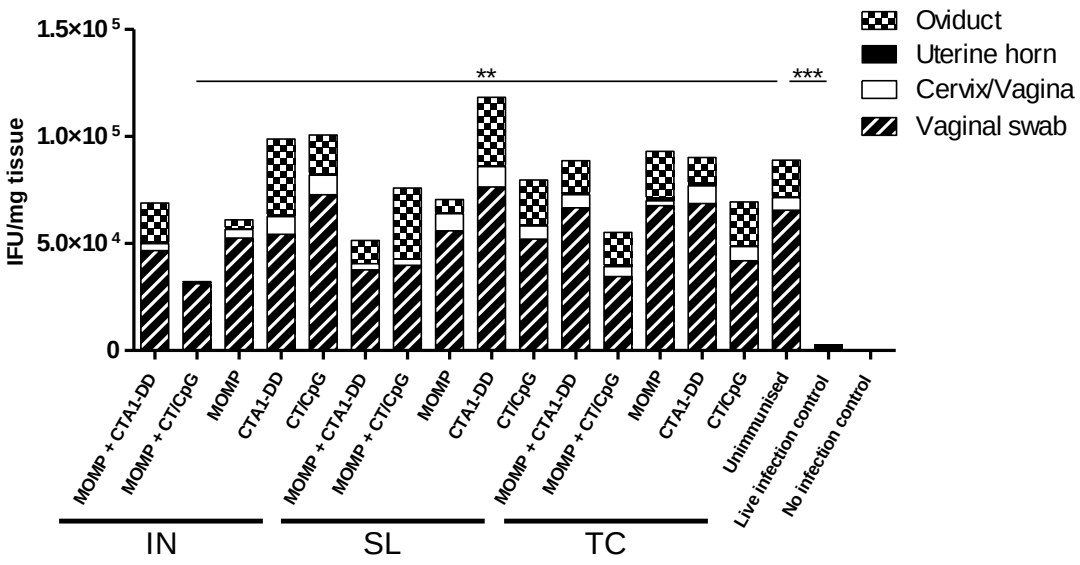


Figure 6.4: Total infectious load detected in the vaginal swab, cervico/vagina, uterine horns and oviducts at day 6 p.i

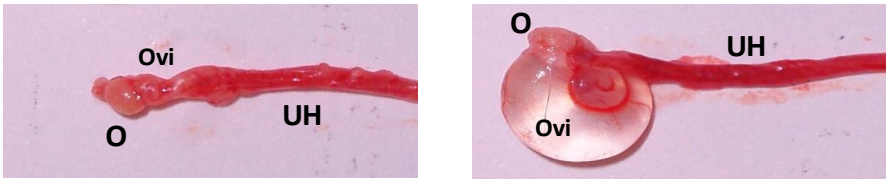
Cervico-vagina, uterine horn and oviduct tissues were taken from all groups at day 6 p.i and homogenised in SPG. The amount of *C. muridarum* in the vaginal swab and each tissue was quantified *in vitro* from the SPG. The chlamydial IFU was determined per swab or per mg of host tissue. Results are presented as the mean only for simplicity. Significant differences were determined for the total; bacterial burden on the entire reproductive tract using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. P >0.05 (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

The effectiveness each vaccine to protect against the pathology that occurs following live *C. muridarum* vaginal challenge

Staining for collagen and hence fibrosis was found to be a poor indicator of pathology as there was often no clear pathological distinction between tissue taken from infected and uninfected groups (Appendix 5). We therefore assessed the incidence and severity of hydrosalpinx on day 49 p.i, as this is a commonly used marker of upper reproductive tract pathology and infertility in the mouse model (Shah *et al.*, 2005b). Following infection, all unimmunised naive animals developed severe uni/bilateral hydrosalpinx, indicated by the significant increase in oviduct diameter when compared to the uninfected control ($P<0.001$) (Figure 6.5). The incidence and severity of hydrosalpinx was similar between the live infection control group and the unimmunised animals. The no infection control group displayed a normal oviduct diameter and no evidence of oviduct pathology. The CT/CpG-containing vaccine delivered via the TC and SL routes, significantly reduced the severity of hydrosalpinx when compared to unimmunised controls ($P<0.01$). Moreover, the incidence of gross pathology was also reduced in contrast to the unimmunised controls by 50%. These animals immunised with the CT/CpG-based vaccine by the SL and TC route, also displayed a reduce peak of infectious burden and shorter durations of infection (Figure 6.2; Table 6.1). Interestingly, IN immunisation with the CT/CpG-containing vaccine appeared to prevent the ascension of infection into the upper reproductive tract (Figure 6.4), however, 75% of animals still displayed evidence of oviduct pathology. The CTA1-DD containing vaccine delivered via the IN route also reduced both the severity and incidence of hydrosalpinx, but this was not found to be significant. The greatest protection against the incidence and severity of hydrosalpinx however was observed following SL immunisation with the CTA1-DD containing vaccine. The severity of hydrosalpinx was reduced significantly by vaccination ($P<0.01$) and 9/10 oviducts were of normal diameter, compared to 2/10 mice in the unimmunised controls (70% reduction). Interestingly, SL application of CTA1-DD-based vaccine, shown to offer the greatest protection from oviduct pathology, did not significantly alter the total burden in tissues at day 6 p.i or infection duration measured in the lower genital tract. This

suggests that the protection against hydrosalpinx conferred by this vaccine was mediated via mechanisms unrelated to bacterial burden.

A.



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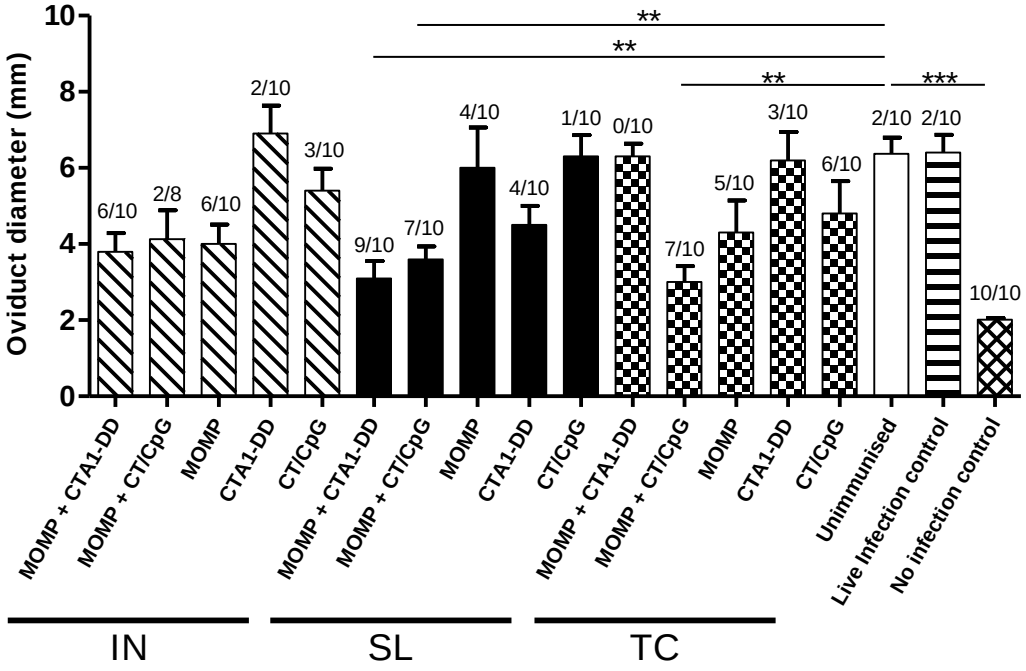


Figure 6.5: Upper reproductive tract pathology determined by hydrosalpinx formation.

Following an IVag challenge with *C. muridarum*, each group of animals was assessed for the development of oviduct pathology post-mortem at day 49 p.i. (A) Shows the appearance of normal (unaffected) uterine horn (UH), oviduct (Ovi) and ovary (O) (left), compared to the gross upper reproductive pathology and oviduct swelling following an IVag infection with *Chlamydia* (right). (B) The severity of pathology was measured by oviduct swelling. The incidence of pathology was represented as a fraction atop of each bar, indicating the number of normal sized oviducts as the numerator, over the total number of oviducts measured as the denominator. Results are presented as the mean ± SD. Each vaccine is grouped with their respective route of immunisation, IN ( ) SL

(■) and TC (▩). Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

Gene expression analysis of oviduct tissues

The development of oviduct pathology in some instances did not reflect the bacterial load cultured from the oviduct tissues, indicating a disconnection between the infection level and disease. We therefore analysed the expression of key factors mediating immunity and immunopathology in the oviduct tissue at day 6 p.i. The day 6 p.i point in time was chosen as it represents the peak of bacterial burden on the oviduct tissue in naive animals (Carey *et al.*, 2009). In addition, treatment of infected animals with antibiotics up to day 7 p.i, eliminates the development of hydrosalpinx (Su *et al.*, 1999), which suggests that the onset of irreversible oviduct pathology begins during or shortly after the peak of infectious burden at day 6 p.i. The oviducts are also where infection-induced inflammation results in tubal blockage and infertility in the mouse. Figure 6.6 depicts the gene expression profiles during a normal course of infection and pathology development (unimmunised), pathology protected (SL delivered MOMP plus CTA1-DD) and the infection protected (IN delivered the MOMP plus CT/CpG), all normalised against the no infection control. In the unimmunised group there was a strong induction of pro-inflammatory cytokines IL-1β (63-fold increase), IL-18 (2-fold increase), TNFα (49-fold increase) and IL-6 (19-fold increase). When this gene expression profile was compared to that of an animal protected from infection exclusively (IN delivered MOMP plus CT/CpG), the infection protected animals displayed a significant down-regulation of pro-inflammatory cytokines IL-1β (62-fold reduction), IL-18 (6-fold reduction), TNFα (48-fold reduction) and IL-6 (13-fold reduction) and up-regulation of Th17-related factors IL-17A (218-fold increase), IL-17C (118-fold increase), IL-17D (11-fold increase), IL-17F (864-fold increase), IL-17RC (4-fold increase), IL-17RD (8-fold increase) and IL-17RE (4-fold increase). Conversely, when a normal course of infection was compared to that of an animal protected from pathology exclusively (SL delivered MOMP plus CTA1-DD), the pathology protected animals displayed a significant up-regulation of Th17-related cytokines IL-17A (165-fold increase), IL-17C (121-fold increase), IL-17D (8-fold increase) and pro-inflammatory cytokines TNFα (5-fold increase) and IL-6 (20-fold increase). In addition, these mice displayed

an increased expression of Th2 and T_{reg}-associated cytokines IL-13 (25-fold increase) and IL-10 (7-fold increase) and the extracellular matrix hydrolysing MMP13 (7-fold increase). These animals also showed a down-regulation of the pro-inflammatory cytokines IL-1β (61-fold reduction) and IL-18 (34-fold reduction), and IL-17 receptors IL-17RD (13-fold reduction) and IL-17RE (136-fold reduction). Noteworthy similarities between both vaccinated groups, was the overwhelming down-regulation of pro-inflammatory cytokines and over-expression of IL-17 cytokines when compared to a normal course of infection. Differences between the vaccines included an increase in IL-13, IL-10, and MMP13 and a decrease in IL-17 receptor expression in the pathology protected group compared to infection protected.

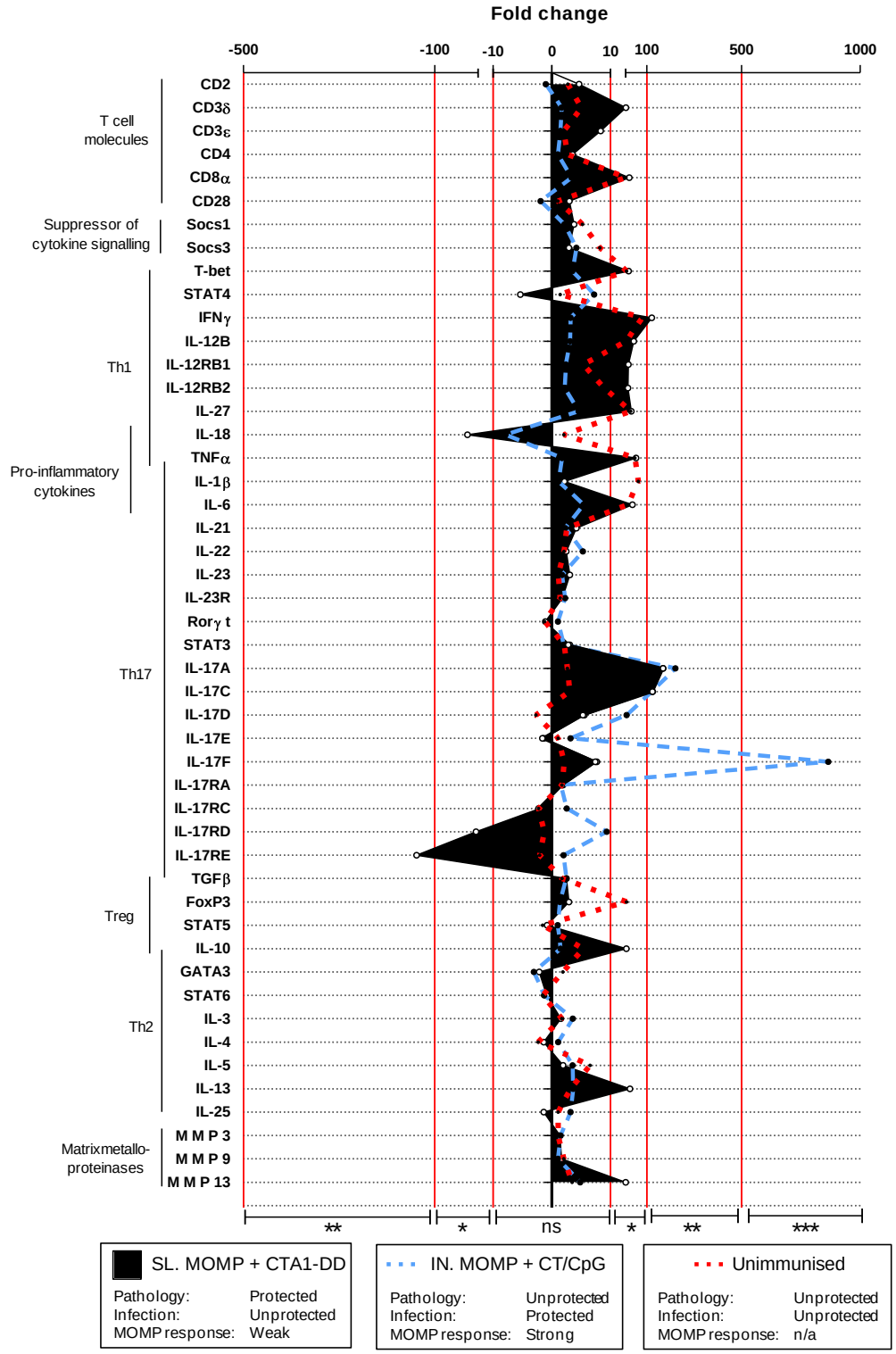


Figure 6.6: Gene expression of key mediators of inflammation and immunity in the oviducts at day 6 p.i.

The RNA was extracted from pooled oviducts from the SL. MOMP + CTA1-DD (pathology – protected; infection – unprotected; MOMP response – weak), IN. MOMP + CT/CpG pathology – unprotected; infection – protected; MOMP response – strong), unimmunised pathology – unprotected; infection – unprotected; MOMP response – n/a) and no infection control groups at day 6 p.i. The difference in expression of T cell

surface receptors, suppressor of cytokine signalling (SOCS), pro-inflammatory cytokines, Th1/Th2/Th17/T_{reg} differentiation factors and MMPs are shown between pathology protected animals, infection protected animals and unimmunised. Expression for all groups were normalised against the no infection control group. Results are presented as the mean fold-change for five mice. Significance was set at >10-fold change. Between 10 – 100-fold-change (*), 100 – 500-fold-change (**) and >500-fold-change (***).

DISCUSSION

The ideal vaccine should elicit immunity against infection as well as pathology. In this chapter, we assessed the level of protection conferred against a chlamydial genital tract infection and the associated disease following immunisation with two different vaccines by three needle-free routes. The CT/CpG adjuvanted vaccine delivered via the SL and TC routes significantly reduced the incidence and severity of pathology, in addition to the duration of infection measured in the lower genital tract. The protection elicited by this vaccine was associated with the induction of antigen-specific secretion of IFNγ and TNFα in the lymph nodes draining the site of infection (Chapter 4), previously shown to elicit protection from infection (Igietseme *et al.*, 1993; Olsen *et al.*, 2010; Yu *et al.*, 2010; Yu *et al.*, 2011; Yu *et al.*, 2012). Despite the induction of antigen-specific antibodies, these appeared to play a minor role in protection against infection and pathology. Interestingly, we also identified two contrasting vaccines capable of enhancing infection resolution or preventing pathology exclusively. The IN delivered CT/CpG-based vaccine prevented the ascension of infection into the oviducts by day 6 p.i, yet the incidence of pathology was unaffected (infection protected). Conversely, the SL delivered CTA1-DD adjuvanted vaccine prevented the development of pathology without significantly altering the bacterial burden in the oviducts (pathology protected). This demonstrated a disconnection between bacterial burden and tissue damage, which presented a unique opportunity to investigate how immunity against infection and pathology can develop independently from one another. In the oviduct tissue, both vaccines suppressed the expression of the IL-1β (implicated in pathology), but displayed opposing effects on the IL-17 signalling pathway. While both vaccines expressed high levels of IL-17 cytokines, the infertility-protected group displayed significantly reduced expression of corresponding IL-17 receptors. This study showed that the degree of protection against infection and tissue damage generated following vaccination is defined by the balance of IL-17-mediated responses.

Immunity against *Chlamydia* genital tract infection is predominantly dependent on the Th1 cell-mediated response, with a minor role for antibodies (Farris and Morrison, 2011). Similarly, the immunity induced by MOMP-based vaccines relies

on the presence of both T cells and antibodies (Farris *et al.*, 2010). B cell-deficient mice are more susceptible to re-infection and shed greater amounts of *Chlamydia* in cervico-vagina secretions between days 5 – 8 p.i following a secondary infection (Su *et al.*, 1997). This suggests that antibodies mediate immunity against the establishment of a genital tract infection and control bacterial burden in the early stages of re-infection. The induction of MOMP-specific antibodies following vaccination did not prevent the establishment of an infection, although there was some minor correlation between serum IgG antibody titres (Figure 4.9) and the control of bacterial burden in the lower genital tract (Figure 6.2, 6.3; Table 6.1). The systemic transfer of MOMP-specific serum or monoclonal antibodies prior to challenge has been shown previously to significantly enhance the resolution of a genital tract infection (Pal *et al.*, 1997b; Farris *et al.*, 2010), contrasting to our findings. However, both of these studies used large and repeated doses of antigen-specific antibodies to confer such strong protection. Furthermore, neither study reported the quantity of antigen-specific antibody reaching the genital tract and whether this amount is physiologically achievable or relevant.

Similar to the respiratory tract model, IgA also played a subordinate role in protection against a genital tract infection. This was likely due to the low levels of IgA detected in the lower genital tract secretions (Cotter *et al.*, 1995), which were similar across all MOMP-immunised groups (Figure 4.11). Therefore, IgA was ineffective at mediating protection against a genital tract infection *in vivo*, possibly due to the induction of insufficient levels of IgA in the mucosal secretions needed to inhibit chlamydial attachment (as shown *in vitro*) (Figure 4.11).

There also appeared to be minimal correlation between the induction of systemic and mucosal IgG titres (Figure 4.9, 4.11, 4.12) and the prevention of oviduct pathology (Figure 6.5). Adoptive transfer of IgG and to a lesser extent IgA, using a backpack hybridoma system reduced the incidence and severity of hydrosalpinx following a genital tract infection (Cotter *et al.*, 1995). The authors suggested that high serum IgG antibody levels together with the highly vascularised upper female reproductive tract lead to a considerable level of antibody transudation from the serum, which contributed to the lower incidence of hydrosalpinx (Cotter *et al.*, 1995). However,

this antibody-mediated protection against upper tract pathology also reduced the chlamydial burden in the oviduct tissues, contrasting to our findings that showed no consistent correlation between antibodies and a reduction in bacterial load in the upper reproductive tract. The overall involvement of antibodies in host defence remains poorly relatable to clearance of a genital tract infection (Johansson *et al.*, 1997) and the role of antibodies in preventing pathology is perhaps even more complex. Similarly, the contribution of antibodies to protection against infection and pathology following a genital tract infection in our study appears minimal.

Sublingual and TC immunisation with the CT/CpG-based vaccine elicited a significant level of protection against the duration of infectious vaginal shedding (Table 6.1) and oviduct pathology (Figure 6.5) following a genital tract challenge. This vaccine induced secretion of the type 1 pro-inflammatory cytokines TNFα and IFNγ by lymphocytes isolated from the MiLN, following *in vitro* stimulation with the MOMP (Figure 4.6). These same stimulated cells also secreted low levels of the inhibitory anti-inflammatory cytokine IL-4. Generating a strong Th1 cell-mediated response is major objective of vaccine design (Yu *et al.*, 2010; Yu *et al.*, 2011; Yu *et al.*, 2012), as $CD4^+$ cells are the dominant contributor toward the resolution of an active infection, immunity against re-infection and protection against upper reproductive tract pathology (Landers *et al.*, 1991; Morrison *et al.*, 1995; Su and Caldwell, 1995; Morrison *et al.*, 2000; Morrison and Morrison, 2001). Induction of IFNγ signalling through the IFNγR is vital for the resolution of a genital tract infection, potentially through both T cell-dependent and independent mechanisms (Cotter *et al.*, 1997b; Perry *et al.*, 1997; Ito and Lyons, 1999; Perry *et al.*, 1999b; Nelson *et al.*, 2005b). The role that TNFα plays is less clear (Darville *et al.*, 2000), but may contribute toward infection resolution as well as pathology (Perry *et al.*, 1999b; Murthy *et al.*, 2011a). Synergism between IFNγ and TNFα has also been reported to enhance the rate of infection clearance more so than either cytokine produced alone (Igietseme *et al.*, 1993; Kannanganat *et al.*, 2007; Olsen *et al.*, 2010; Yu *et al.*, 2010; Yu *et al.*, 2011; Yu *et al.*, 2012). This may occur by increasing cytokine production, antigen-presentation and recruitment of T cells into the genital tract (Perry *et al.*, 1998; Fichorova and Anderson, 1999; Gabr *et al.*, 2011). Therefore, the induction of IFNγ and TNFα following immunisation coincided with

strong protection against a genital tract infection, potentially due to a synergistic interaction between multiple pro-inflammatory cytokines. To our knowledge, this is the first evidence of this combination cytokine response conferring protection against oviduct pathology following a genital tract challenge.

Furthermore, it was essential that these antigen-specific and pro-inflammatory cells were positioned in the local lymph nodes draining the genital tract (MiLN) to enhance protection. This is an identical finding to that in the respiratory tract infection model (Chapter 5), where the induction of pro-inflammatory cytokine responses in MdLN and not the spleen, predicted protection against infection and disease. Proliferation of antigen-specific T cells, the acquisition of effector function and migratory properties can be detected in the regional lymph node draining the site of a HSV infection up to 2 days prior to any response in the spleen (Coles *et al.*, 2002). Similarly, Roan *et al*., (2006) showed the importance of MiLN-resident *Chlamydia*-specific T cells for the induction of protective immunity in mice against an intrauterine challenge with the human *C. trachomatis* serovar L2. Following the adoptive transfer of *Chlamydia*-specific T cell antigen 1 (Cta1) NR1 $CD4^+$ T cell clones; these cells were found to proliferate extensively in the MiLN 3 – 4 days following the infection. These T cells were found to express higher levels of activation markers (CD69 and CD44) and low levels of CD62L, indicative of tissue-homing effector memory phenotype. Seven days following the infection, NR1 cells were detected in the genital tract tissues, expressing the same activation and effector memory phenotype identified on NR1 cells proliferating in the MiLN. This response conferred an equivalent amount of protection against chlamydial burden to that seen in the strongly resistant live infection control group. Importantly, the same level of proliferation and activation of NR1 cells could not be detected in non-draining lymph nodes (Roan *et al.*, 2006), which may indicate that the antigen-specific cells localised in the MiLN and not other peripheral lymphoid tissues are major drivers of protection against a *Chlamydia* genital tract infection in mice. Therefore, protection against the magnitude of infection and the development of oviduct pathology following a chlamydial genital tract infection relies heavily on priming and positioning type 1 cytokine-secreting cells in the regional lymph nodes draining the site of infection, potentially for the initiation of a rapid response to infection.

Immunisation with the CT/CpG-adjuvanted vaccines via the SL and TC routes may have promoted the expression of homing receptors on activated lymphocytes and enhanced their recruitment to distant mucosal effector sites via the CMIS.

Oviduct pathology however (Figure 6.5), was not always directly linked with the bacterial burden in the upper reproductive tract (Figure 6.4). The infection protective vaccine (IN. MOMP plus CT/CpG) appeared to prevent the ascension of infection into the oviducts, but not the development of pathology when compared to the unimmunised naive mice. Conversely, the pathology protective vaccine (SL. MOMP plus CTA1-DD) reduced the incidence of oviduct pathology to near that seen in uninfected mice without significantly altering the bacterial burden in the oviducts. This disconnection between bacterial burden and the development of pathology was also seen in the respiratory tract model of infection (Chapter 5) and has been reported elsewhere in the literature (Maxion *et al.*, 2004; Carey *et al.*, 2009; Wang *et al.*, 2009). Increasing the inoculation doses of *C. muridarum* modulates the innate and adaptive response; however the level of gross oviduct pathology that develops is not significantly different between animals given 5×10^2 verses 1×10^7 IFU intravaginally (Maxion *et al.*, 2004; Carey *et al.*, 2009). This has also been shown in the context of a vaccine, where IM immunisation with T3SS protein Tarp together with IFA/CpG provided almost no protection against the infection measured in the lower genital tract, but significantly reduced the incidence of hydrosalpinx (Wang *et al.*, 2009). We therefore decided to investigate the cause of this anomaly in more detail, by analysing the gene expression in the oviduct tissues, directly involved in pathogenesis, between the infection- and pathology protected groups. Although analysis at multiple points in time would have been desirable, we chose day 6 p.i for analysis as it represents the peak bacterial burden in the oviducts and corresponds to a period in which the onset of irreversible pathology begins in naive animals (Su *et al.*, 1999; Carey *et al.*, 2009). The most notable difference in gene expression following infection between vaccinated and unvaccinated animals was an up-regulation of pro-inflammatory cytokines IL-1β and IL-18 in the unimmunised group. Macrophages and neutrophils are the major source of IL-1β and IL-18, but these cytokines are also produced by *Chlamydia*-infected epithelial cells (Munder *et al.*, 1998; Lu *et al.*, 2000a; Hook *et al.*, 2005; Prantner *et al.*, 2009). This is

consistent with the finding that the immune cells recruited into the oviduct around day 6 following a primary infection of naive mice are predominantly innate (macrophages and neutrophils), as opposed to adaptive (T and B cells) (Kelly and Rank, 1997; Kelly *et al.*, 2000). Therefore, the immune response of an unimmunised animal to a chlamydial infection in the upper reproductive tract, at a point in time consistent with the development of irreversible pathology, was predominantly driven by innate immunity.

Both IL-1β and IL-18 were significantly up-regulated in the infected unimmunised group by comparison to vaccinated groups (Figure 6.6). Interestingly, caspase-1 cleaves pro-IL-1β and pro-IL-18 to the mature and biologically active forms (Thornberry *et al.*, 1992), which may indicate the involvement of the inflammasome in the development of pathology in naive animals. Capase-$1^{-/-}$ mice exhibit a normal primary and secondary course of infection, yet these animals develop significantly less pathology than WT mice (Cheng *et al.*, 2008), suggesting that caspase activity is in part responsible for the development of pathology in naive mice. The cytokine IL-18 is yet to be investigated in the genital tract model of infection, although IL-18-deficiency has been shown to have minimal effect on the clearance of infection and pathological damage following a lung infection (Lu *et al.*, 2000b). Interleukin-1β on the other hand has been extensively implicated in the development of pathology following a genital tract chlamydial infection (Cheng *et al.*, 2008; Prantner *et al.*, 2009; Nagarajan *et al.*, 2012), even in the absence of infiltrating leukocytes (Hvid *et al.*, 2007). Infected unvaccinated animals displayed over a 60-fold up-regulation of IL-1β compared to both vaccinated groups, at a point in time corresponding with the commencement of irreversible pathology (Su *et al.*, 1999). Consistent with the literature (Cheng *et al.*, 2008), IL-1β appears to play a dominant role in oviduct pathogenesis in naive animals during a primary infection, which may be due to a greater reliance on innate immunity (neutrophils and macrophages) for the clearance of infection (Figure 6.7).

Both vaccinated groups however showed a stronger down-regulation of IL-1β when compared to the unvaccinated group, which may indicate a shift in these animals to utilise the adaptive immune response generated following vaccination opposed to the

damaging innate response. However, the infection protected vaccine group developed pathology, despite the reduction in IL-1β expression. This suggests that there are mechanisms in addition to IL-1β/caspase-1, contributing towards pathology in mice that may involve an immunopathological adaptive element.

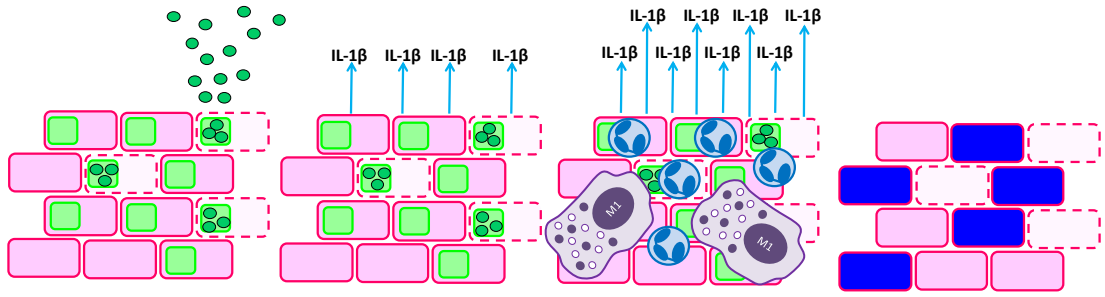
The most notable difference in gene expression between the infection protected and unvaccinated groups regarding aspects of the adaptive immune response was the increased expression of Th17-realated constituents. The infection protected vaccine group displayed enhanced expression of IL-17 cytokines (IL-17A, C, D and F) and their associated receptors (IL-17RC, D and E) compared to the unimmunised group, indicating a potential increase in IL-17-mediated signalling. The cytokine IL-17A (also known as IL-17) is crucial for host defence against numerous intracellular pathogens (Khader *et al.*, 2009), including *Chlamydia* (Bai *et al.*, 2009). Interleukin-17 and IL-17F are produced primarily by the $CD4^+$ Th17 subset of T cells (Dong, 2008) and influence the recruitment of neutrophils and development of Th1 immunity (Bai *et al.*, 2009; Watanabe *et al.*, 2009; Scurlock *et al.*, 2010). Furthermore, Yu *et al*., (2010) recently described a correlation between $IFN\gamma^+IL\text{-}17^+$ $CD4^+$ T cells and enhanced resolution of chlamydial genital tract infection. The infection protected group, shown to have elevated expression of IL-17 cytokines and their associated receptors in the oviducts, cleared the infection in the upper reproductive tract significantly faster than unimmunised animals that displayed almost no expression of Th17-related factors. Our data therefore supports a protective role for IL-17-mediated defence against a *Chlamydia* genital tract infection, at least in relation to infectious burden.

However, $IFN\gamma^+IL\text{-}17^+$ $CD4^+$ T cells, deemed protective against chlamydial infection (Yu *et al.*, 2010; Yu *et al.*, 2011; Yu *et al.*, 2012), are also enriched in tissue affected in Crohn's disease and experimental autoimmune encephalomyelitis (EAE) (Neurath *et al.*, 2002; Bettelli *et al.*, 2004), suggestive of a link between Th17 and autoimmunity. Lu *et al*., (2011) also recently found that protection from a chlamydial infection following vaccination associated with high levels of IFNγ, yet a reduction in pathology required decreased levels of IL-17. As IL-17-mediated responses have been implicated in both immunopathology and host defence against pathogens

(Peters *et al.*, 2011), this may explain why the infection protected group was unable to prevent oviduct pathology despite preventing the infection ascension. Supportive of this theory of vaccine-induced IL-17-mediated protection and immunopathology, the pathology protected vaccine group displayed decreased expression of IL-17 cytokines and IL-17 receptors, signifying a potential inhibition of IL-17-mediated signalling that may have prevented the development of oviduct pathology and the rapid clearance of infection.

The precise mechanism(s) behind IL-17-mediated immunity and immunopathology in regards to a chlamydial infection are unknown. Interleukin-17 cytokines can act upon epithelial cells as the mucosa highly expresses IL-17 receptors (Li *et al.*, 2006; Ramirez-Carrozzi *et al.*, 2011). The cytokine IL-17 does not have a direct anti-chlamydial effect at least *in vitro* (Zhang *et al.*, 2009) and may instead confer immunity by recruiting and activating of other adaptive immune populations (Bai *et al.*, 2009). Alternatively, stimulation of epithelial cells with IL-17 induces the release of pro-inflammatory cytokines, chemokines and various colony-stimulating factors that encourage the recruitment of tissue-damaging neutrophils (Fossiez *et al.*, 1996). Interleukin-17 cytokines further synergises with pro-inflammatory cytokines like IL-1β and TNFα produced by the epithelia cells (Jovanovic *et al.*, 1998; Yamaguchi *et al.*, 2007; Gaffen, 2009; Onishi *et al.*, 2010; Chiricozzi *et al.*, 2011) to support the longevity of the pro-inflammatory immune response. This response may recruit and activate additional macrophages and neutrophils, producing NO and MMPs (Prause *et al.*, 2004) that have been shown to be damaging during a chlamydial genital tract infection (Dixon *et al.*, 2010; Lee *et al.*, 2010b; Frazer *et al.*, 2011). Moreover, IL-1β and TNFα can stimulate a further release of IL-17 cytokines and expression of IL-17 receptors (Chang *et al.*, 2011; Ramirez-Carrozzi *et al.*, 2011). Therefore, the induction of IL-17-mediated responses following vaccination may create a positive feedback loop, potentially initiated by the adaptive response and sustained by the host cell epithelium (Figure 6.7), not unlike the cellular hypothesis of chlamydial pathogenesis (Stephens, 2003).

A.



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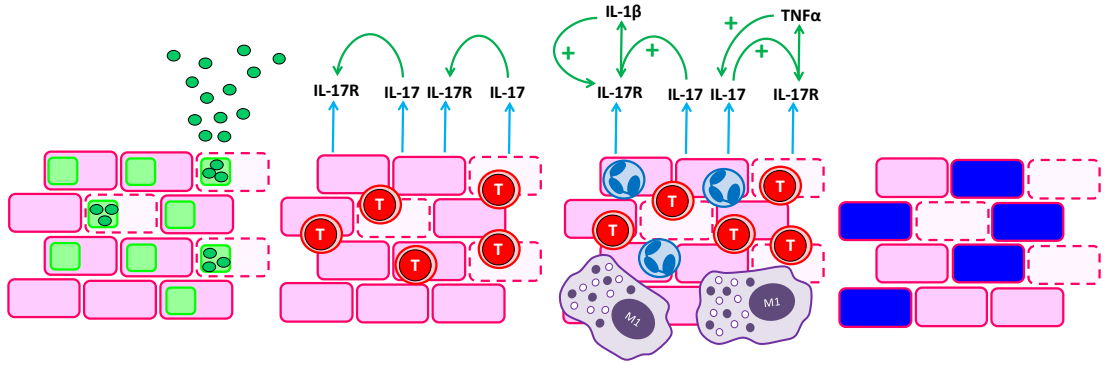

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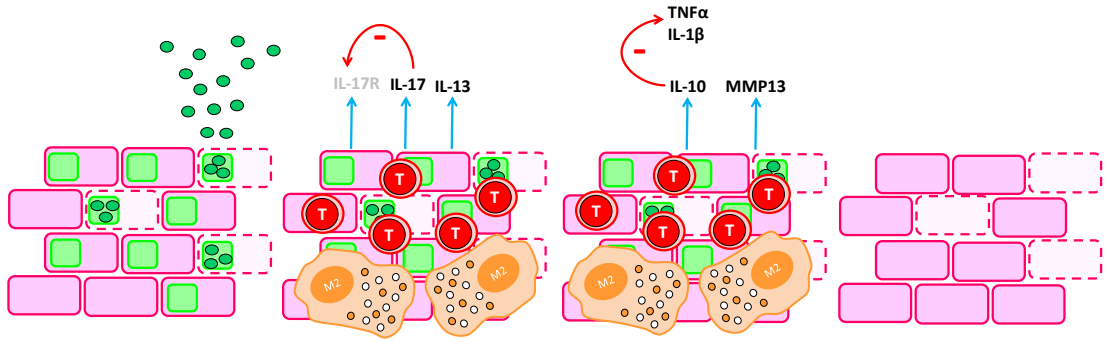

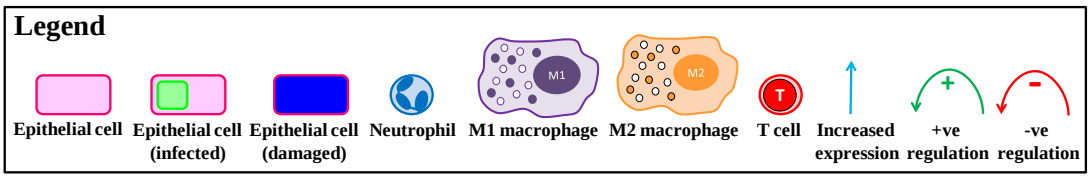

Figure 6.7: Hypothesised mechanisms of infection clearance and development of pathology in the oviducts in vaccinated and unvaccinated animals.

(A) Infection of naive animals leads to a significant induction of pro-inflammatory cytokines, mainly tissue damaging IL-1β. This could lead to an influx of neutrophils and M1 macrophages (SOCS1<SOCS3), which are the major producers of IL-1β. Over-production of IL-1β leads to direct tissue damage. (B) Infection of the infection protected group (IN. MOMP plus CT/CpG) leads to a significant up-regulation of IL-17-mediated

defences, which rapidly resolves the infection. Prolonged IL-17 signalling may however lead to the recruitment of neutrophils and secretion of pro-inflammatory cytokines by epithelial cells. Pro-inflammatory cytokines recruit M1 macrophages (SOCS1<SOCS3) and increase IL-17-mediated defences, which causes tissue damage. (C) Infection of the pathology protected group leads to a significant up-regulation of IL-17 cytokines, but not the receptors required for optimal signal transduction or eradication of the infection. IL-13 is also expressed which activates anti-inflammatory M2 type macrophages (SOCS1>SOCS3). M2 macrophages secrete IL-10 and MMP13 involved in inhibiting pro-inflammatory cytokine secretion and enhancing tissue repair, respectively.

The role of IL-17 signalling was recently assessed in the context of a primary *Chlamydia* genital tract infection (Scurlock *et al.*, 2010). The IL-17RA is an essential co-receptor to the majority of IL-17 receptors, necessary for optimal signal transduction of IL-17A, C, D and F (Kuestner *et al.*, 2007; Rong *et al.*, 2009; Hu *et al.*, 2010; Chang *et al.*, 2011; Ramirez-Carrozzi *et al.*, 2011). Mice deficient in IL-17RA therefore largely portray the involvement IL-17 signalling plays in host defence and disease. The IL-17RA$^{-/-}$ mice were found to resolve a primary infection and develop pathology similar to WT animals (Scurlock *et al.*, 2010). For the most part, this study recognised IL-17-mediated responses as redundant in the development of oviduct pathology following a primary infection. However, pathology mediated by IL-17 signalling reported in our study was due to the presence of an adaptive response generated following vaccination and not present during a primary infection. Therefore, we expand on the findings by Lu *et al.*, (2011) implicating IL-17 signalling, driven by the adaptive immune response, as playing a causative role in upper reproductive tract pathology following a chlamydial genital tract infection.

How each vaccine was able to differentially regulate IL-17-mediated responses is unclear, however, the route of immunisation (Zygmunt *et al.*, 2009) and adjuvants (McNeal *et al.*, 2007; Hasselberg *et al.*, 2009; Meza-Sanchez *et al.*, 2011; Xu *et al.*, 2012) utilised may have contributed. The infection protective vaccine contained both CT and CpG that elicit high levels of TGF-β, IL-6 and IL-17 (Meza-Sanchez *et al.*, 2011; Xu *et al.*, 2012), which can facilitate the development of Th17 immunity. These adjuvants however also promote Th1 and Th2 immune responses, which are known suppressors of Th17 responses (Weaver *et al.*, 2006). The IN route utilised by the infection protective vaccine may also have been a contributing factor. Zygmunt *et*

al., (2009) described a predisposition for immunisation via the IN route to promote Th17 immune responses due to an adjuvant-independent over production of IL-6 by DC localised in the NALT. Alternatively, the SL mucosa targeted by the pathology protective vaccine, which displayed reduced IL-17 responses, may elicit a more tolerogenic phenotype. The human oral mucosa, displaying similar cellular distribution to BALB/c mice (Mascarell *et al.*, 2009), expresses high levels TGF-β and FoxP3 and low levels of Th17 differentiation factors IL-6 and RORγt and associated cytokines IL-17A, IL-17F, IL-22 and IL-26 (Allam *et al.*, 2011). CTA1-DD also elicits a high level of IFNγ with relatively low levels of IL-17 (McNeal *et al.*, 2007), identical to the type of response that is responsible for the protection against pathology (Lu *et al.*, 2011). In this study, the oviduct tissues of animals immunised with the CTA1-DD adjuvanted vaccine via the SL route displayed a higher expression of IFNγ and IL-10 compared to the infection protected group, cytokines previously shown to negatively regulate Th17 immunity (Harrington *et al.*, 2005; Cruz *et al.*, 2006; Chaudhry *et al.*, 2011; Huber *et al.*, 2011; Lazarevic *et al.*, 2011). The IL-10 cytokine has also been shown *in vitro* to inhibit the production of potentially damaging pro-inflammatory cytokines by epithelial cells and macrophages (Yilma *et al.*, 2012), which may be important in controlling the development of pathology in the upper reproductive tract tissues. Although speculative, the IL-17-mediated bias induced within animals protected from infection, may have been the result of utilising the IN route and CT/CpG adjuvant combination. Conversely, the SL route, the CTA1-DD adjuvant and increased expression of IFNγ and IL-10 in the oviduct tissue, may have suppressed the induction of IL-17-mediated defence in pathology protected animals.

In addition to the potential suppression of IL-17-mediated signalling, the phenotype of the pathology protected group could also be explained by the activation of M2 macrophages that inhibit inflammation and mediate wound repair. Activated by IL-4 and IL-13 secreted by $CD4^+$ Th cells (Loke *et al.*, 2007), M2 macrophages are a major source of IL-10 and MMP13. Interestingly, IL-13, IL-10 and MMP13 are significantly up-regulated in oviducts of the pathology protected group when compared to the infection protected vaccine group. Alternatively activated macrophages (M2) differ from classical macrophages (M1) by the synthesis of

arginase, which inhibits NO production used by M1 macrophages to kill intracellular bacteria (Briken and Mosser, 2011). As NO production has both protective and pathological roles during a *Chlamydia* infection (Ramsey *et al.*, 2001a; Ramsey *et al.*, 2001b; Jayarapu *et al.*, 2010), this may also explain the phenotype of the pathology protected vaccine, protected from the development of oviduct disease but not the infection. In mice, the ratio of SOCS1 and SOCS3 can be used to identify the induction of M1 and M2 macrophages, where the SOCS1:SOCS3 ratio is high in M2 macrophages but low in M1 macrophages (Dickensheets *et al.*, 2007; Whyte *et al.*, 2011). In the present study, the SOCS1:SOCS3 ratio favoured the M2 phenotype in the oviducts of the pathology protected animals (3:2), but polarised towards the M1 type for both the unvaccinated (2:3) and infection protected vaccine group (1:2). This was also found to be the case during a *N. brasiliensis* lung infection, where M2 macrophages facilitated rapid resolution of tissue damage (Chen *et al.*, 2012). Therefore, the over-expression of M2-related cytokines and MMPs, in addition to a SOCS's ratio that favours the development of arginase-expressing M2 macrophages, may implicate M2 macrophages mediating inflammation and wound repair in the pathology protected vaccine group.

CONCLUSIONS

The aim of this chapter was to assess the level of protective immunity against a *Chlamydia* genital tract infection and the associated disease following immunisation with the MOMP plus CTA1-DD or CT/CpG via TC, IN and SL routes. The MOMP plus CT/CpG vaccine was most protective against a reproductive tract infection and associated pathology when delivered by the SL and TC routes. Intranasal immunisation with the MOMP plus CTA1-DD was partially protective against infection, yet SL administration with the same vaccine provided near complete protection against pathology. The immune responses induced following immunisation, determined in Chapter 4, were then compared with the level of protection against infection and disease. Although there appeared to be a minor correlation between the induction of serum IgG and protection, antigen-specific production of TNFα and IFNγ in the lymph nodes draining the site of infection (MiLN) were found to have the strongest connection with early infection resolution and pathology prevention. This study also indicated that the IL-17-mediated responses generated following vaccination can determine the balance between infection control and tissue damage. An over-expression of IL-17 signalling may facilitate the early eradication of infection but also exacerbate pathology, potentially through the induction of a cyclical pro-inflammatory response. Therefore, the induction of IL-17-mediated responses should be avoided for an effective vaccine against a *Chlamydia* genital tract infection, by careful selection of adjuvants and immunisation routes. We also provided the first, albeit circumstantial evidence, of the involvement of M2 type macrophages potentially modulating protection against *Chlamydia*-induced oviduct pathology.

CHAPTER SEVEN: GENERAL DISCUSSION AND FUTURE DIRECTIONS

Chlamydia is rife in the community and infections are responsible for a wide range of diseases with enormous global economic and health burden. To date, screening and treatment strategies employed by many countries have failed to stem the rise in prevalence long-term and as a result the development of an effective vaccine is the only viable alternative. *Chlamydia* is a pathogen that infects through mucosal surfaces, therefore protection is reliant on generating mucosal immunity. Effective induction of mucosal immunity occurs best when targeting vaccination to the epithelium, which stimulates APCs present in the mucosal tissues that are specialised in initiating responses to mucosal pathogens. The success of vaccines targeting the mucosal epithelium is reliant on a potent adjuvant to elicit robust activation and maturation of APCs present at the site of immunisation, necessary to overcome the induction of tolerance. A major obstacle to the development of an effective vaccine for *Chlamydia* is the lack of proven strong adjuvants that induce good protection at mucosal surfaces, without harmful side effects. We addressed this gap in two ways, by investigating a novel non-toxic mucosal adjuvant (CTA1-DD) and by utilising different immunisation routes (IN, SL and TC) to limit the toxicity of adjuvants precluded from human use (CT and CpG), while still harnessing their immunogenicity.

The CTA1-DD adjuvant was safe and immunogenic via the IN route

Currently, there is only one commercially available vaccine that targets a mucosal surface other than the gut. FluMist® is a trivalent live-attenuated influenza vaccine delivered intranasally, which elicits a greater level of local immunity in the lungs than the more commonly used inactivated influenza vaccine delivered parenterally (Brokstad *et al.*, 2002). The live attenuated nature of this vaccine means it is self-replicating and -adjuvanting and does not require the addition of a potent mucosal adjuvant to elicit protective immunity. Nasalflu® however, containing the inactivated flu virosome, required the addition of the powerful enterotoxic LT adjuvant to elicit a protective response following IN delivery. As a direct result of the adjuvant, some patients developed CNS complications following IN vaccination (Fujihashi *et al.*, 2002; Couch, 2004; Kiyono and Fukuyama, 2004; Mutsch *et al.*, 2004). This side effect was substantiated by an additional clinical trial using two different IN subunit vaccines which also contained the LT adjuvant (Lewis *et al.*,

2009). The IN route is of particular interest for human vaccine design against mucosal pathogens as it targets the Waldeyer's tonsillar ring, which contain unique mucosal inductive sites, specialised in promoting trafficking of mucosal immunity to distant effector sites like the reproductive and respiratory tracts (Kiyono and Fukuyama, 2004; Brandtzaeg, 2009). As most subunit vaccines targeted to the epithelium require a potent adjuvant to generate a protective response at the mucosal surface, there is a need for more effective adjuvants that are safe when delivered by the IN route. In the present study, CTA1-DD was found to be both immunogenic and safe by the IN route in mice. The emergence and success of novel non-toxic adjuvants like CTA1-DD make the nasal route safer to target for vaccination, which has profound implications for human vaccines developed against mucosal pathogens.

The CT/CpG adjuvant was safe and immunogenic via needle-free routes of immunisation other than IN

Cholera toxin and CpG have historically been the "gold standard" mucosal adjuvants for use in animal studies, as these prevent mucosal tolerance and elicit strong immune responses following vaccination. The inherent toxicity of both CT and CpG has restricted their use in humans, particularly via certain routes of immunisation like IN (Fujihashi et al., 2002; Couch, 2004; Heikenwalder et al., 2004; Kiyono and Fukuyama, 2004; Mutsch et al., 2004; Klinman et al., 2007; DeFrancesco, 2008). Utilising different immunisation routes can however limit the toxicity of certain adjuvants in both animals and humans, while still harnessing their immunogenicity (Skelding et al., 2006; McKenzie et al., 2007; Frech et al., 2008). In the present study, CT/CpG was both safe and immunogenic by the SL and TC routes in mice. This has significant implications for human vaccine development for a number of reasons. Needle-free immunisation targeting the epithelium is preferred to injections as it is safer and more cost-effective (if the threat of blood-borne diseases is considered), does not require trained personnel, facilitates rapid immunisation of large populations and enhances the population compliance critical for herd immunity (Giudice and Campbell, 2006). Overall, adjuvants initially thought to be too toxic for human use, may be administered via SL or TC route and still maintain effectiveness needed for the development of needle-free vaccines capable of inducing mucosal immunity.

The protection elicited by SL and TC immunisation with the CT/CpG-based vaccine could have a real benefit to the community, if resistance can be replicated in humans

Induction of complete sterilising immunity against *Chlamydia* is most desirable, but may be difficult or impossible to achieve (Beagley *et al.*, 2009b). Altering the course of infection may be a more realistic measure of controlling infection and reducing disease. Gray *et al*., (2009) recently mathematically modelled the potential impact of vaccines with different efficacies against *C. trachomatis*, using behavioural, biological and clinical data. It was suggested that implementing a vaccine that elicited even moderate gender-specific protection i.e. reduced chlamydial shedding or infection duration, predominantly targeting females, has the potential to completely eradicate all infection and *Chlamydia*-related sequelae from the human population (Gray *et al.*, 2009). The acquired immunity generated following SL and TC immunisation with CT/CpG in conjunction with the MOMP, was protective against both genital and respiratory tract *Chlamydia* challenges, reducing both the peak and duration of infection. According to the models used by Gray *et al.*, (2009), this vaccine could have a positive influence on infection and disease prevalence if the results can be replicated in humans.

Vaccination by the SL and TC routes with the CT/CpG-based vaccine also reduced the subsequent development of oviduct pathology in female mice by 50%. It is estimated that the Australian health systems spends between $90 – 160 million each year on *C. trachomatis*-related illness, with disease in women accounting for 80% of the total outlay (Patel *et al.*, 2008). In addition to reducing reproductive tract pathology, the CT/CpG-based vaccine delivered via SL and TC routes also reduced the fibrotic scarring and lung consolidation associated with the exacerbation of COPD and asthma. Chronic lower respiratory tract disease like COPD and asthma were the underlying cause of 6,122 deaths in 2010 (ABS, 2010), with the total annual economic cost of over $100 billion in Australia (NAC, 1992; ALF, 2010). A chlamydial vaccine that provides simultaneous protection against infection and pathology at both mucosal surfaces has enormous implications for global health, if protection can be translated from mice to humans.

Mouse models of infection are an essential first step for pre-clinical development and testing of vaccines. However, a number of key differences exist between rodents and humans, in the context of the vaccination strategy used in this study, which will require further investigation in animal models more akin to human anatomy and physiology. Rodent skin, targeted by TC immunisation, is considerably more permeable with more hair follicles than human skin. These differences in the epithelia architecture may influence crucial passage of the vaccine into the epithelium and hence immunogenicity. Alternatively, the oral cavity of rodents, targeted by SL immunisation, is more keratinised compared to humans (Song *et al.*, 2009). This could affect dosing by the rate or amount of absorption through the buccal mucosa. Saliva contains various levels of esterases, carbohydrases and phosphatases, which could also prove to reduce a vaccine's immunogenicity due to normal digestive processes (Sohi *et al.*, 2009). A long-acting sedative like ketamine, used in animals to facilitate SL immunisation and prevent swallowing, reduces salivary secretions and potentially dampens any affect saliva has on a vaccines efficacy (Moench *et al.*, 2003). Saliva production and composition also varies based on the animal model, disease state and the nature of the vaccine, and as a result a saliva reducing agent may be needed to accompany any vaccine delivered SL to prevent enzymatic interference (Siegel, 1984). Sublingual vaccines have been used successfully to target allergy in humans, although the activity loss attributed to saliva needed to be considered when determining the appropriate dose (Igea *et al.*, 1994).

Mice are also considerably less susceptible to the gastrointestinal effects of CT compared to humans, who develop overt diarrhoea when exposed to as 5µg of CT (Levine *et al.*, 1984). It is probable that topical immunisation with CT will be non-toxic in humans (McKenzie *et al.*, 2007; Frech *et al.*, 2008). However, administration of CT to the oral cavity, during SL immunisation, has the potential to enter the gut and cause diarrhoea in humans, toxicity that would not have been observed in the resistant rodent model. Furthermore, CpG could be less immunogenic in humans compared to mice, if a vaccine is to be targeted to the epithelium. TLR9, the target of CpG, is expressed by the majority of mDCs and other innate cell types present at each immunisation site in mouse. The TLR9 molecule in humans however is only expressed by B cells and pDCs that are not routinely found in the epithelium (Krieg,

1995; Klinman, 2004). The CpG adjuvant applied to the epithelium of humans may therefore be less immunogenic than in mice, due to the differential expression of TLR9 by APCs. Although the effectiveness of both the adjuvants (CT/CpG) and routes (SL and TC) reported in the present study using the rodent model is not directly applicable to humans, the success of these vaccination strategies justifies further examination in an animal more comparable to humans.

The non-human primate is the best pre-clinical model to evaluate vaccines, although a single animal can cost upwards of $100,000(USD) to care for over its life-time (Nath *et al.,* 2000). Alternatively, pigs share approximately 78% homology with human genes regulating immunity and inflammation (Dawson, 2012). Swine also have comparable skin and oral cavity architecture (Sullivan *et al.,* 2001; Sohi *et al.,* 2009) and distribution of immune cells in the epidermis to humans (Fritz *et al.,* 1990; Bos, 2005), making the porcine model appropriate for assessing the potential efficacy of both the SL and TC routes of immunisation in humans. The porcine model of human *C. trachomatis* genital tract infection has also recently been developed (Schautteet and Vanrompay, 2011), which would enable a live challenge with a human serovar of *C. trachomatis* to determine the level of protective vaccine-induced immunity against infection *in vivo*. While the pig model would provide a more human-like model for the testing the efficacy of each route of immunisation, it may not accurately reflect the safety and immunogenicity of the CT/CpG adjuvant combination to that in humans. Swine share the same resistance to the enterotoxogenic effects of CT and expression of TLR9 as rodents (Foss and Murtaugh, 1999; Balmelli *et al.*, 2010). Therefore, any successful trial in pigs would require further optimisation in the non-human primate model. In the primate model, the use of the CT adjuvant via the SL route may require the development of a controlled release device, similar to those used in pharmakinetics studies (Senel *et al.,* 2012), to minimise the risk of the enterotoxin entering the gastrointestinal tract. Due to the likely reduced ability of the CpG adjuvant to elicit an immune response from APC residing in the epithelium of primates, an alternate Th1-polarising adjuvant may need to be implemented before a human vaccine can be developed. Progress towards a needle-free human chlamydial vaccine utilising the SL or TC

route will require controlled delivery and adjuvant selection in order to maintain the vaccine's safety and immunogenicity.

Another limitation of most animal models is the live *Chlamydia* challenge and how it reflects protection against infection transmission in the human population. The MOMP-based vaccines may elicit some cross protection against other serovars (Zhang *et al.*, 1987; Zhang *et al.*, 1989), although the MOMP usually generates serovar-specific immunity that is primarily directed towards four highly variable surface exposed domains (Ramsey *et al.*, 2009). This means that immunisation with the MOMP commonly confers protection only against a homologous strain of *Chlamydia* expressing an identical MOMP antigen. This is also the case in the present study, where the MOMP administered during vaccination and the MOMP expressed by the strain of *C. muridarum* used to challenge share an identical amino acid sequence encoded by a matching *omp*A gene. As multiple *C. trachomatis* serovars circulate within the human population, many with diverse *omp*A genotypes containing nonsynonomous mutations, the current mouse model cannot assess protection against heterologous challenges due to the limited number of mouse-specific pathogens. Future studies in pigs or primates could allow for heterologous challenge experiments with a number of different human serovars. There are however two potential solutions to counteract the induction of serovar-specific immunity in the human population, a polyvalent or multi-subunit vaccine approach. The polyvalent pneumococcal vaccine Pneumovax 23® utilises 23 variants of the same antigen to provide maximum population coverage against *Streptococcus pneumoniae* serotypes common in the geographical region. A polyvalent vaccine containing MOMP antigens derived from serovars E, F and G could protect against 90% of *C. trachomatis* infection in Australia (Mossman *et al.*, 2008). Alternatively, a multi-subunit approach incorporating a number of chlamydial antigens particularly those highly conserved among *Chlamydiales* could protect against multiple serovars (Stagg, 1998; Li *et al.*, 2007). With the availability of both *C. trachomatis* and *C. pneumoniae* genomes, identification of such novel vaccine candidates is occurring on a much larger scale. Although the current mouse model cannot simulate the problem of serovar-specific immunity following vaccination, there are strategies that can be employed to prevent this occurring in humans.

The longevity of immunity induced following vaccination is also critically important for population-based protection against *Chlamydia* (Gray *et al.*, 2009). Most prophylactic vaccines require repeated booster immunisations to maintain immunity against infection. This would also likely be the case for any human *Chlamydia* vaccine, particularly if the vaccine was comprised of subunits. Induction of long-lasting immunity has been reported for both the adjuvants (CT and CpG) and routes (SL and TC) (Naito *et al.*, 2007; Klinman *et al.*, 2010; Negri *et al.*, 2010), although this was not investigated in the present study. Future experiments will need to monitor the persistence of antigen-specific responses and immunity to infection after extended periods following vaccination. Despite the inherent limitations present when choosing any animal model, the successful induction of protective immunity by the MOMP plus CT/CpG vaccine in mice justifies a trial in other animal models more comparable with humans.

***Induction of a strong pro-inflammatory cell-mediated response in lymph nodes regional to the site of infection was the best associate of protection against* Chlamydia**

The frequency and/or magnitude of lymphocytes producing IFNγ is commonly used as the major indicator of protective immunity against *Chlamydia*. However, the induction of multi-functional $TNF\alpha^{+}IFN\gamma^{+}CD4^{+}$ Th cells following vaccination was shown to be a better associate of protection than single cytokine-producing cell types (Olsen *et al.*, 2010; Yu *et al.*, 2010; Yu *et al.*, 2012). In the present study, we consistently detected MOMP-specific production of TNFα and IFNγ by cells isolated from the lymph nodes draining the reproductive and respiratory tracts in experimental groups strongly protected from both infectious challenges. Secretion of TNFα by $CD8^{+}$ T cells has however been found by others to exacerbate pathology (Murthy *et al.*, 2011a). Therefore, it is vital that future studies assess the cytokine secretion profiles of individual $CD4^{+}$ and $CD8^{+}$ T cells following vaccination. This may provide better predictors of protective immunity and enable the design of future vaccines capable of inducing multi-functional T cell phenotypes.

Priming and positioning antigen-specific cells in the lymph nodes draining the sites of infection and not the spleen, was vital for optimal protective immunity against

pathology following both genital and respiratory tract challenges. The importance of generating an immune response in the regional lymph nodes as opposed to the spleen following vaccination in order to elicit protection against infection has not previously been reported in the context of a *Chlamydia* infection. This has significant ramifications for chlamydial vaccine design as most research focuses on splenic responses, which may prove to be a poor indicator of immunity particularly against mucosal pathogens (Kundig *et al.*, 1996; Bachmann *et al.*, 1997; Bachmann *et al.*, 2005; Santosuosso *et al.*, 2005; Roan *et al.*, 2006; Forbes *et al.*, 2008; Judy *et al.*, 2012; Mackay *et al.*, 2012). It is unclear whether the protection conveyed by antigen-specific lymphocytes residing in the lymph nodes is a consequence of earlier initiation and recall of immunity or if a distinct memory population is present with a superior ability to home to the mucosal tissues following activation. Many studies have shown that surgical or genetic deletion of lymph nodes impairs the development of immunity against infection and disease (Rayhane *et al.*, 1999; Lee *et al.*, 2000; Kumaraguru *et al.*, 2001; Lund *et al.*, 2002; Vonk *et al.*, 2002; Kallal *et al.*, 2010). However, protection against some pathogens can be increased in the absence of lymph nodes (Magez *et al.*, 2002). Therefore, future studies may utilise $LT\alpha^{-/-}$ or lymphadenectomised mice to assess the importance of secondary lymphoid tissues in initiating infection resolution and protection against pathology following a primary *Chlamydia* infection.

Phenotyping and adoptive transfer studies of spleen verses lymph nodes could also be used to determine if these cells home faster and have mucosal/central memory/effector memory characteristics. Congenic mice could be used to assess immune recall. Thy1.1/1.2 or Ly5.1/5.2 congenic mice, express a unique T cell allele that can be detected with an antibody to differentiate host and donor lymphocytes. Isolation of T cells from the lymph nodes or spleens of Thy1.1 congenic mice recovering from a chlamydial infection could be adoptively transferred into separate naive Thy1.2 mice. A subsequent infection of the recipient Thy1.2 mice may be used to determine the recruitment time of spleen- verses lymph nodes-derived lymphocytes based on the appearance of the Thy1.1 antigen at the site of infection. This future direction may reaffirm the idea that circulating memory T cells possess a limited ability to localise and control infection in peripheral tissues (Kundig *et al.*,

1996; Bachmann *et al.*, 1997; Bachmann *et al.*, 2005). Further work may also analyse the expression of activation and homing molecules on antigen-specific cells in the draining lymph nodes verses the spleen following vaccination. Identification of a unique homing phenotype could enable a vaccination strategy to be tailored to recruit antigen-specific responses into the lymph nodes as well as the mucosal tissues, ideal for protection against *Chlamydia*.

A reduction in the bacterial load does not always associate with protection against pathology.

Protection against a *Chlamydia* infection requires the induction of a robust pro-inflammatory cytokine response. However, it is believed that the tissue damage associated with a chlamydial infection is not a direct effect of bacterial colonisation or bacterial load, but an indirect consequence of a pathological pro-inflammatory immune response vital to eradicate the infection (immunopathology). The present study identified a disconnection between bacterial burden and development of disease following both genital and respiratory tract challenges, which supports the theory that the magnitude of inflammation following infection and not the bacterial load is mediating the severity of disease. We investigated this finding in more detail in the genital tract model of infection. The production of pro-inflammatory cytokines, mainly IL-1β, was associated with development of disease in unvaccinated animals. However, the balance of IL-17-mediated defence appeared to determine the level of protection against pathology in vaccinated animals. This suggested that there may be at least two mechanisms driving disease following a *Chlamydia* genital tract infection, one driven by the innate response through IL-1β/caspase-1 and the other by the Th17 adaptive immunity generated following vaccination. This has profound implications for future chlamydial vaccines, as currently many vaccines are designed to maximise the pro-inflammatory response (including Th17 responses) to improve infection clearance, with minimal consideration given to protection against pathology. This finding has identified a potential problem with the current focus of chlamydial vaccine design, particularly if the ultimate goal is to reduce the incidence of disease. Therefore, identifying the contribution of the Th17 response towards protection and immunopathology is crucial for the development of more efficacious vaccines.

The role the Th17 response plays during a chlamydial infection is only just starting to be unravelled. There is evidence in the literature to suggest that IL-17 signalling plays no role in immunity or disease following a primary infection (Scurlock *et al.*, 2010). High levels of Th17 responses generated during vaccination however have been implicated in both infection resolution and immunopathology following a genital tract infection (Yu *et al.*, 2010; Lu *et al.*, 2011). A similar dual role of IL-17 signalling was also found in the present study, where over-expression of IL-17 cytokines and their associated receptors in the upper reproductive tract of vaccinated animals were linked with the enhancement of infection resolution and adverse oviduct pathology following an IVag challenge. The mechanism behind IL-17-mediated protection and immunopathology was speculated to be attributed to a later recruitment of damaging neutrophils and macrophages. However, gene expression and immunohistochemical analysis of the oviducts at different points in time are needed to validate our hypotheses and further investigate the role of IL-17-mediated responses. Future studies may also expand on the work by Scurlock *et al.*, (2010) by adoptively transferring $CD4^+$ T cells from immune WT mice into $IL\text{-}17RA^{-/-}$ mice to examine the role IL-17 signalling plays during a secondary genital tract infection. Alternatively, immunisation with the Th17-polarising adjuvant β-glucan curdlan (LeibundGut-Landmann *et al.*, 2007) together with the MOMP prior to challenge could also be used to assess whether Th17-driven immunity exacerbates pathology. As the Th17 response is known to have different roles following both genital and respiratory tract challenges (Bai *et al.*, 2009; Zhang *et al.*, 2009; Yu *et al.*, 2010; Lu *et al.*, 2011), the proposed future studies will also need to be repeated using the respiratory tract model. These studies may clarify the protective and pathological roles of the Th17 response during a *Chlamydia* infection and whether it should be avoided following vaccination.

Alternately activated macrophages (M2) were also implicated in mediating tissue repair and reducing the incidence of hydrosalpinx following a genital tract challenge in the present study. This was demonstrated by the overexpression of IL-13, IL-10, MMP13 and SOCS1>SOCS3 ratio. M2 type macrophages are thought to be inducers of tolerance as opposed to immunopathology (Gordon, 2003). Mice with dysfunctional M2 type macrophage have been shown in other models to display

delayed wound healing and excessive fibrosis (Wilson and Wynn, 2009). Evidence for the presence of M2 type macrophages suggests the existence of a Th2-driven anti-inflammatory response (Loke *et al.*, 2007). T helper type 2 cells are widely believed to be non-protective (Perry *et al.*, 1997; Wang *et al.*, 1999; Yang, 2001; Hawkins *et al.*, 2002) and contribute to pathology following a primary infection by inhibiting the development Th1 immunity (Yang *et al.*, 1996; Perry *et al.*, 1997; Wang *et al.*, 1999; Kaiko *et al.*, 2008; Chen *et al.*, 2010; Asquith *et al.*, 2011). However, in the presence of strong Th1 immunity generated following a primary infection, Th2 cells may serve to minimise the potential inflammatory damage inherent with Th1 over-expression (Buendia *et al.*, 2002), potentially by activating M2 type macrophages. Future studies may search for further evidence of M2 macrophages during a natural infection by staining for arginase. In addition, the adoptive transfer of $SOCS1^{-/-}$ (M1) or $SOCS3^{-/-}$ (M2) monocytes into diphtheria toxin (DT) treated CD11bDTR mice (depleted of macrophages), may also unveil a regulatory function of macrophages following a genital tract challenge. The involvement of M2 type macrophages has not been reported previously and presents an exciting new avenue to study chlamydial pathogenesis.

In conclusion, an efficacious vaccine against *Chlamydia* may require the stimulation of mucosal immunity. Of importance is the development of potent yet safe delivery systems or mucosal adjuvants capable of overcoming tolerance and eliciting long-lived immunity against infection. Our hypothesis was that utilising different immunisation routes and novel non-toxic mucosal adjuvants will elicit simultaneous protection against *Chlamydia* genital and respiratory tract infections, without any harmful side effects. In the present study, we have shown that the non-toxic mucosal adjuvant CTA1-DD is safe and immunogenic via the IN route. Although too dangerous for use in a human in prophylactic vaccine delivered intranasally, CT/CpG was also found to be both safe and immunogenic if alternate routes of administration (SL and TC) were utilised. Moreover, the MOMP adjuvanted with CT/CpG also possesses the potential to have an enormous impact on *C. trachomatis*- and *C. pneumoniae*-related burden, if protection can be successfully translated into the human population. Furthermore, our study identified a disconnection between bacterial load and disease severity, challenging the current premise that pathology

can be prevented by simply reducing the level of infection. Investigation of this anomaly shed light on novel mechanisms of chlamydial pathogenesis, driven by the adaptive immune response generated following vaccination. This study illustrates the challenges involved when developing a vaccine against *Chlamydia* and forces us to rethink how we design future vaccines.

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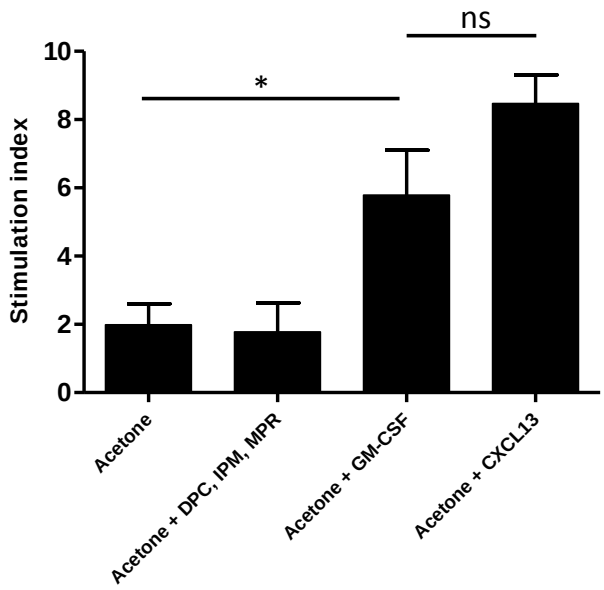
APPENDIX

APPENDIX ONE

A.



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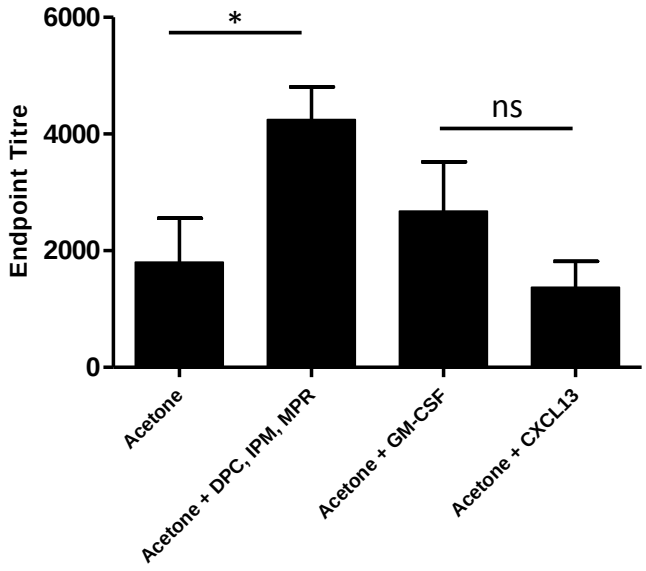


Figure A1: Antigen-specific proliferation and serum IgG antibody titres following different skin pre-treatments prior to TC immunisation.

The skin of each group of animals was pre-treated with a combination of permeation enhancers (acetone or DPC, IPM, MPR) and permeation enhancers (GM-CSF or CXCL13) prior to being immunized with the MOMP plus CTA1-DD via the TC route. (A) The spleens were excised from vaccinated animals, pooled and homogenised to create a single-cell suspension. Splenocytes were stimulated with the MOMP or media for 72 hr and then incubated with thymidine for an additional 14 hr. Cell proliferation was determined by incorporation of thymidine into newly synthesised DNA. The cpm value (ionising radiation) of the MOMP stimulated cells was divided by the cpm value obtained from the media stimulated cells to give a stimulation index. (B) The induction of MOMP-specific serum IgG following vaccination was quantified by ELISA. Endpoint titres were calculated for all samples using background absorbance of PBST plus two SD. Results are presented as the mean

± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

APPENDIX TWO

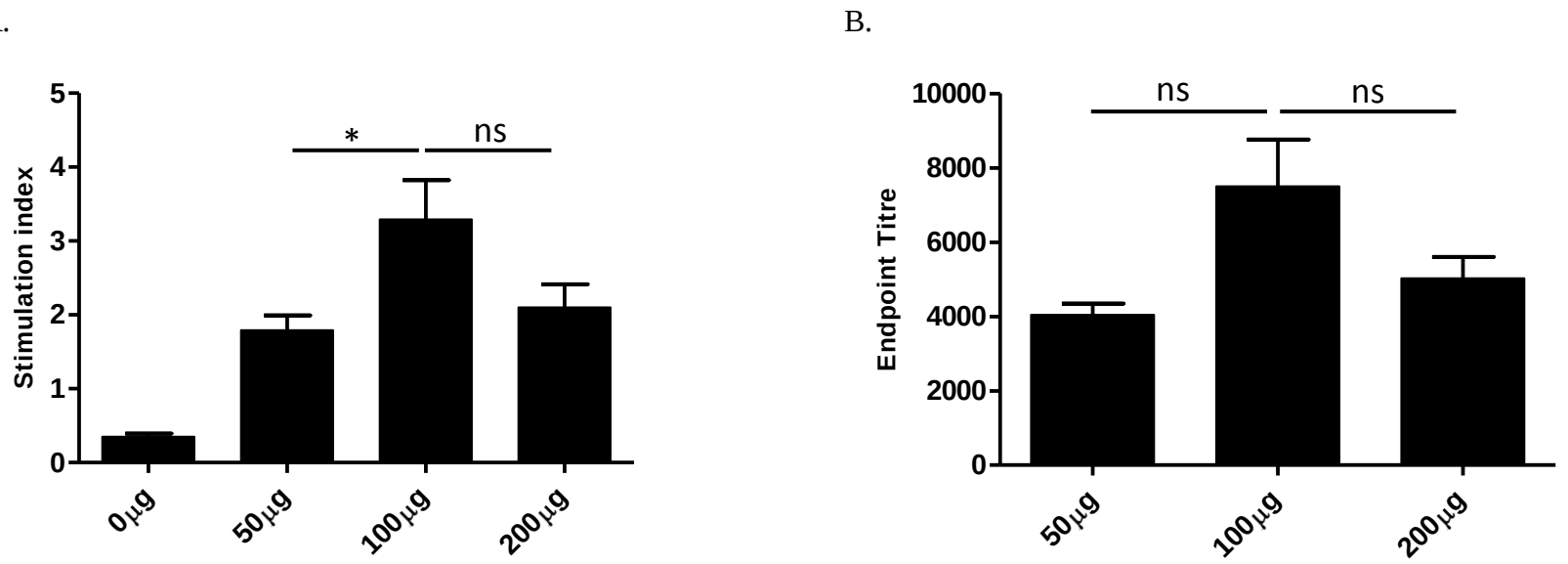
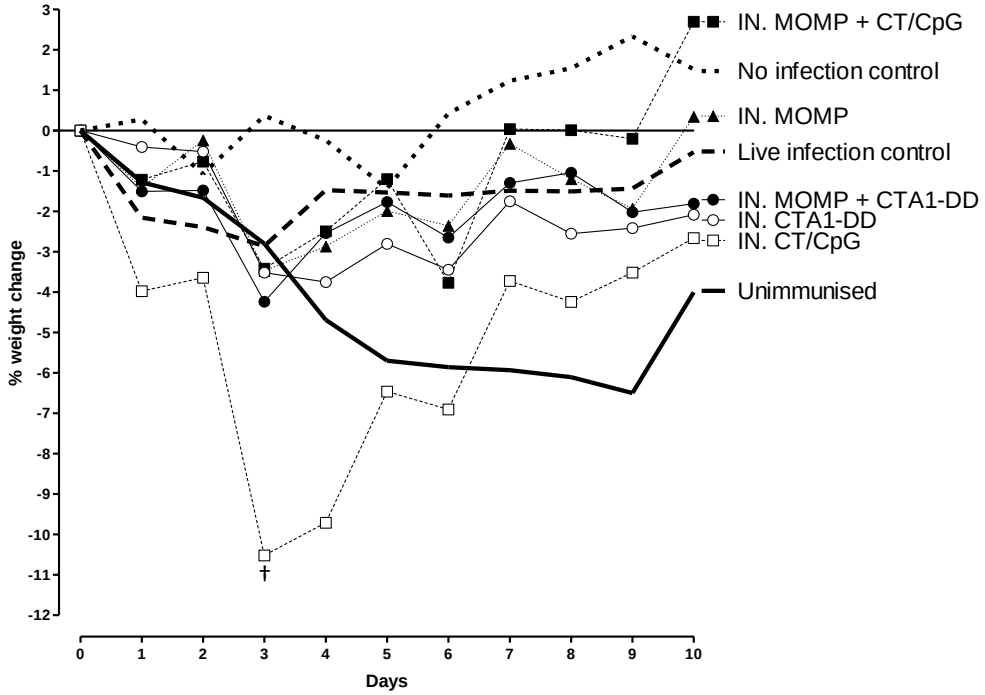


Figure A2: Antigen-specific proliferation and serum IgG antibody titres following SL immunisation with 50, 100 and 200µg of the MOMP.

The optimal antigen dose following SL immunisation was determined by administering 50, 100 and 200µg of the MOMP together with CTA1-DD to the buccal mucosa. (A) The spleens were excised from vaccinated animals, pooled and homogenised to create a single-cell suspension. Splenocytes were stimulated with the MOMP or media for 72 hr and then incubated with thymidine for an additional 14 hr. Cell proliferation was determined by incorporation of thymidine into newly synthesised DNA. The cpm value (ionising radiation) of the MOMP stimulated cells was divided by the cpm value obtained from the media stimulated cells to give a stimulation index. (B) The induction of MOMP-specific serum IgG following vaccination was quantified by ELISA. Endpoint titres were calculated for all samples using background absorbance of PBST plus two SD. Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

APPENDIX THREE

A.



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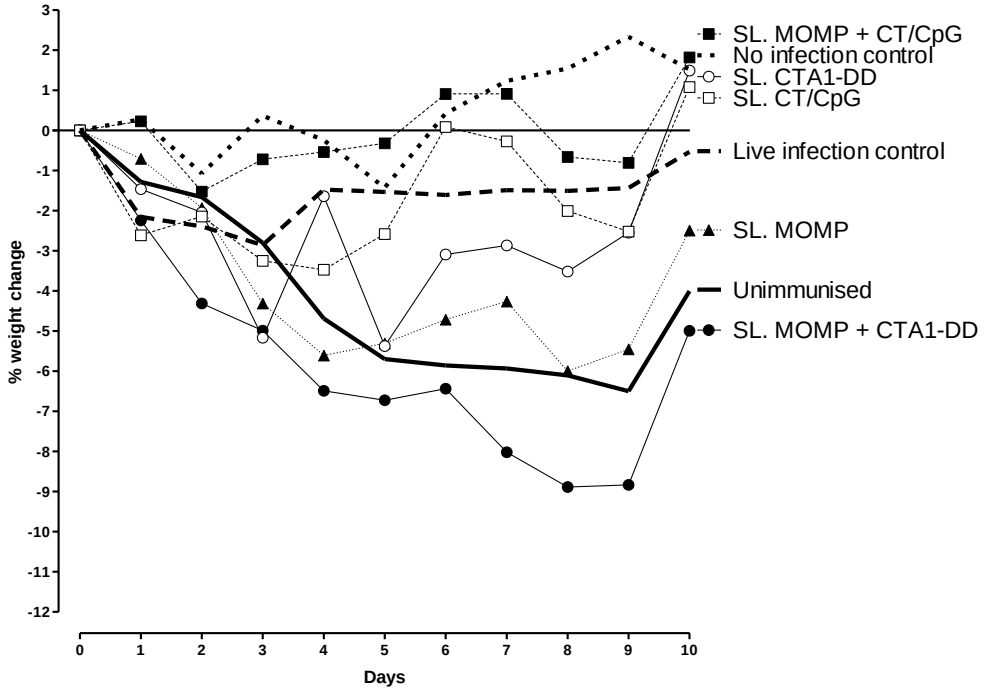

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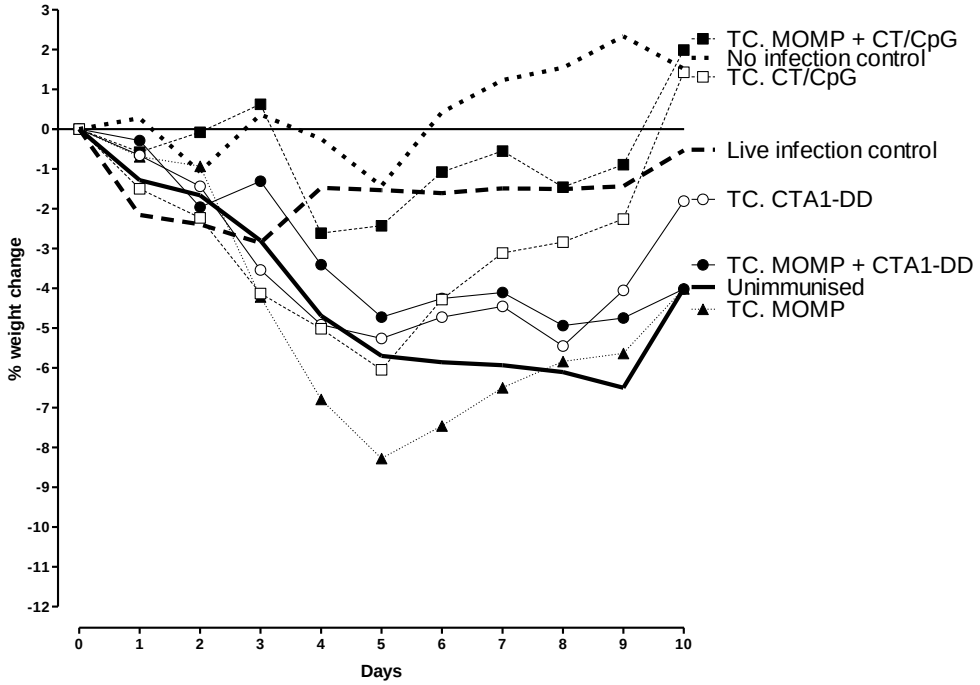


Figure A3: Percentage weight change of all groups animals following IN challenge with *C. muridarum*.

Each mouse was weighed prior to infection to establish a starting weight and daily following infection to determine weight change. Percentage weight change was for each animal was calculated by comparing the pre-infection body weight to daily p.i body weights. This figure depicts the effect each vaccination strategy has on weight change following an IN infection. Both CT/CpG- and CTA1-DD-based vaccines delivered via the (A) IN, (B) SL and (C) TC route are grouped together to show the effect each adjuvant has on protection. Antigen, adjuvants, unimmunised (primary infection), live infection (secondary infection) and no infection controls are also included. Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test by comparing the weight changes between groups at the same point in time. One *P* value, the most significant, is given for groups showing a significant change. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

APPENDIX FOUR

A.

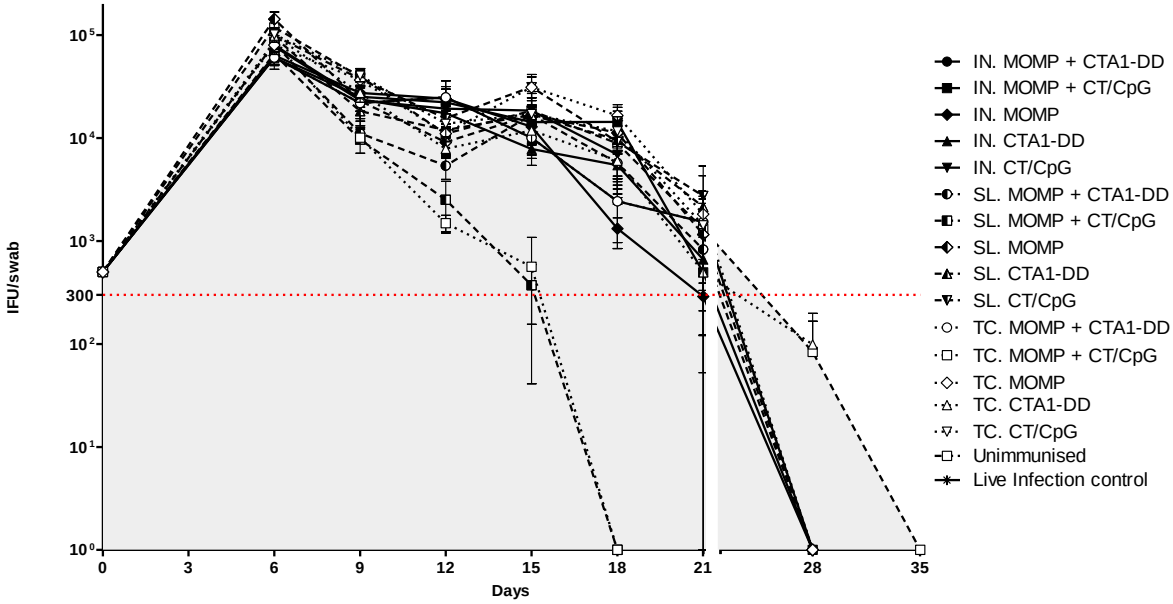
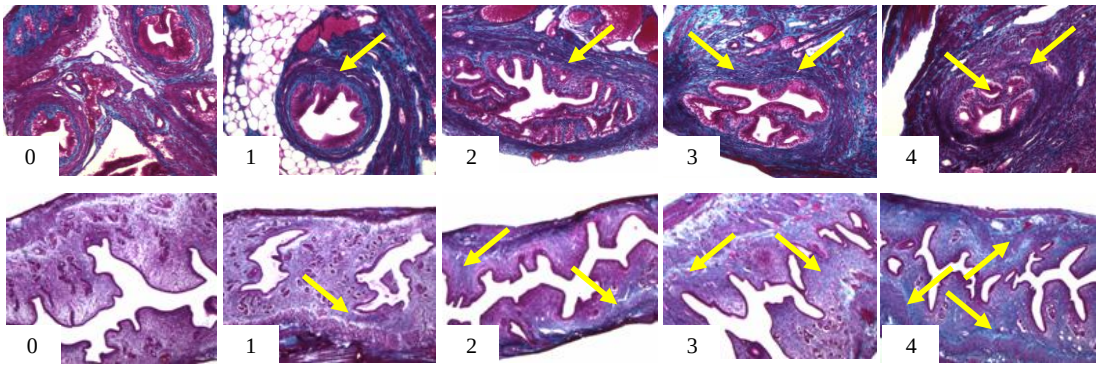


Figure A4: Chlamydial shedding into the vagina collected by the vaginal swab of all groups animals following IVag challenge with *C. muridarum*.

Following an IVag challenge with *C. muridarum*, vaginal swabs were collected over the entire duration of an infection and stored in SPG. The amount of *C. muridarum* collected by each swab was quantified *in vitro* from the SPG. Animals were deemed to have productive infection at ≥300IFU/swab. Cut-off level for a productive infection was set at ≥300IFU/swab. Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

APPENDIX FIVE

A.



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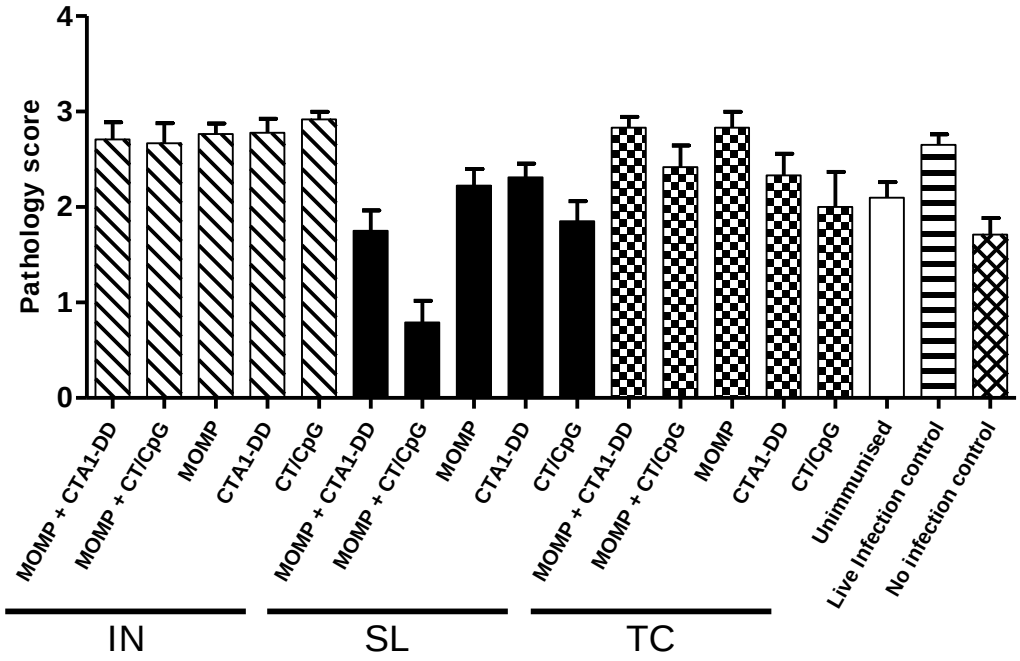

Figure A5: Fibrotic scarring of the uterine horns and oviducts following IVag infection with *C. muridarum*.

Uterine horn and oviduct tissues taken from each mouse at day 49 p.i were paraffin embedded, sectioned and stained for fibrosis using Masson's trichrome. (A) The pathology score scale used to compare the development of disease following infection. Representative histological images of the oviducts (top panel) and uterine horns (bottom panel) for each pathology scores are depicted. "0" signifies healthy, undamaged tissue. Scores of "1" through to "4" depict worsening degrees of collagen deposition (blue) and the beginnings of obstruction (yellow arrows). (B) Combined pathology score from uterine horns and oviducts was assigned by three separate individuals blinded to groups and experimental design. Results are presented as the mean ± SD. Each vaccine is

grouped with their respective route of immunisation, IN (, SL (and TC ( ). Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

APPENDIX SIX

Accession No.	Symbol	Fold-change		
		IN. MOMP + CT/CpG	SL. MOMP + CTA1-DD	Unimmunised
NM_009786	Cacybp	1.12	1.12	-1.11
NM_011329	Ccl1	1.09	3.64	1.82
NM_011333	Ccl2	**13.23**	**113.21**	**92.25**
NM_016960	Ccl20	2.73	**41.28**	**12.45**
NM_009137	Ccl22	1.14	-1.38	1.60
NM_013654	Ccl7	6.31	**34.68**	**25.02**
NM_013486	Cd2	-1.03	4.39	2.73
NM_031162	Cd247	1.01	-2.04	-3.59
NM_007642	Cd28	-1.89	2.77	1.06
NM_133654	Cd34	-1.40	-1.83	-1.91
NM_013487	Cd3d	1.67	**10.84**	5.02
NM_007648	Cd3e	1.48	8.07	2.05
NM_009850	Cd3g	2.36	4.50	2.23
NM_013488	Cd4	1.00	3.29	2.68
NM_011616	Cd40lg	3.05	5.58	2.44
NM_001081110	Cd8a	3.40	**25.87**	**11.34**
NM_009883	Cebpb	1.54	3.02	2.47
NM_020008	Clec7a	3.10	**10.54**	5.91
NM_009969	Csf2	1.60	2.83	2.33
NM_009971	Csf3	2.11	5.06	3.10
NM_009142	Cx3cl1	1.36	2.28	1.77
NM_008176	Cxcl1	**13.17**	**188.55**	**85.73**
NM_021704	Cxcl12	1.11	-1.06	1.08
NM_009140	Cxcl2	5.34	**307.75**	**199.65**
NM_009141	Cxcl5	**17.84**	**208.16**	**232.74**
NM_080729	Il25	3.20	-1.60	1.10
NM_007901	S1pr1	-1.49	-1.55	-1.29
NM_054039	Foxp3	1.40	2.70	**14.40**
NM_008091	Gata3	-3.04	-2.39	1.89
NM_010493	Icam1	-1.02	7.09	6.18
NM_017480	Icos	1.32	**10.82**	8.03
NM_008337	Ifng	3.20	**120.37**	**80.01**

NM_010548	Il10	1.52	**12.65**	5.33
NM_008352	Il12b	3.13	**44.22**	**12.52**
NM_008353	Il12rb1	2.40	**21.89**	5.34
NM_008354	Il12rb2	2.21	**19.64**	8.89
NM_008355	Il13	3.64	**28.52**	3.16
NM_008357	Il15	**-57.34**	**-24.14**	3.06
NM_010552	Il17a	**220.62**	**167.74**	2.65
NM_145834	Il17c	**121.08**	**123.85**	3.03
NM_145837	Il17d	**14.06**	5.32	-2.71
NM_145856	Il17f	**865.95**	7.43	2.07
NM_008359	Il17ra	1.54	1.70	1.75
NM_019583	Il17rb	-1.10	-2.79	-2.15
NM_134159	Il17rc	2.55	-2.26	-2.25
NM_134437	Il17rd	9.36	**-14.45**	-1.29
NM_145826	Il17re	2.00	**-138.27**	-2.06
NM_008360	Il18	-7.87	**-32.19**	2.06
NM_008361	Il1b	1.18	1.92	**63.25**
NM_008366	Il2	1.29	3.13	2.06
NM_021782	Il21	2.27	3.93	2.56
NM_016971	Il22	5.32	2.24	2.08
NM_031252	Il23a	1.74	2.82	1.09
NM_144548	Il23r	2.28	1.58	1.45
NM_145636	Il27	4.56	**33.66**	**31.35**
NM_010556	Il3	3.59	1.32	1.72
NM_021283	Il4	1.11	-1.62	-2.31
NM_010558	Il5	3.54	1.68	6.54
NM_031168	Il6	5.43	**39.13**	**18.89**
NM_010559	Il6ra	1.28	1.84	1.29
NM_008372	Il7r	1.03	4.07	1.92
NM_020583	Isg20	1.77	3.09	2.35
NM_146145	Jak1	-1.05	1.05	-1.34
NM_008413	Jak2	2.22	3.54	1.51
NM_008607	Mmp13	4.85	**10.39**	3.48
NM_010809	Mmp3	1.53	1.13	1.07
NM_013599	Mmp9	1.06	1.43	2.04
NM_010851	Myd88	3.16	8.30	3.82

NM_010899	Nfatc2	1.48	1.54	1.15
NM_008689	Nfkb1	1.14	1.48	1.43
NM_011281	Rorc	1.04	-1.33	-1.20
NM_009896	Socs1	2.18	**18.72**	5.23
NM_007707	Socs3	4.20	**21.84**	8.18
NM_011486	Stat3	2.06	2.82	2.10
NM_011487	Stat4	7.24	-5.60	1.44
NM_011488	Stat5a	1.03	-1.04	-1.54
NM_009284	Stat6	-1.28	-1.12	-1.56
NM_011518	Sykb	1.21	3.81	3.22
NM_019507	Tbx21	3.50	**22.93**	**14.15**
NM_011577	Tgfb1	2.47	2.25	1.74
NM_054096	Tirap	1.05	2.00	-1.27
NM_021297	Tlr4	1.36	1.19	-1.00
NM_013693	Tnf	1.74	**53.83**	**49.26**
NM_009424	Traf6	1.67	1.26	1.93
NM_009537	Yy1	1.12	-1.23	-1.29
NM_010368	Gusb	-1.32	-1.47	-1.01
NM_013556	Hprt	1.50	1.22	1.19
NM_008302	Hsp90ab1	-1.31	-1.31	-1.32
NM_008084	Gapdh	1.26	1.36	-1.09
NM_007393	Actb	-1.09	1.16	1.23

Figure A6: Complete list of genes, accession numbers and fold-changes of genes regulating inflammation and immunity.

RNA was extracted from pooled oviducts from the SL. MOMP + CTA1-DD (pathology – protected; infection – unprotected; MOMP response – weak), IN. MOMP + CT/CpG pathology – unprotected; infection – protected; MOMP response – strong), unimmunised pathology – unprotected; infection – unprotected; MOMP response – n/a) and no infection control groups at day 6 p.i. Expression for all groups were normalised against the no infection control group. Results are presented as the mean fold-change for five mice.